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Journal of Embryology and Experimental Morphology

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Alkaline Phosphatase Activity in the Rat Uterus[1]

by K. V. PRAHLAD *and* C. H. CONAWAY[2]

From the Department of Zoology, University of Missouri

A COMPARATIVE study of the distribution of placental scars from the first and second pregnancies in laboratory rats indicated that the most posterior scar of first pregnancy tends to be nearer to the cervix than the most posterior second pregnancy scar (Momberg & Conaway, 1956). Frazer (1955) in an earlier study observed that more embryos implanted in the caudal half of the uterus of the rat than in the cranial half when the embryo number was four or less. Momberg & Conaway (1956) found this difference to be greater when the embryo number was high.

The reasons for such distributions are not known, but these findings suggest that the uterus varies along its length and does not provide a uniform environment for the implanting embryos. It would further appear that analyses of the various regions of the uterus might furnish information about conditions offering favourable and less favourable environments for the implantation of the blastocysts. Conspicuous alterations in total mass and composition of the rat uterus during the estrous cycle are well established. However, relatively little is known about regional differences in the uterus. An observation regarding differences between regions of the same uterus has been made by Hayashi *et al.* (1957). Their histochemical studies revealed that the B-glucuronidase activity varied considerably, not only among individuals, but among blocks cut from different portions of the same uterus.

Even such physiologically important enzyme systems as the phosphatases have not been studied in a manner that would determine local differences in various regions of the same uterus. Phosphatases have been identified in all animal tissues and are now known to be concerned in many important processes such as carbohydrate metabolism, nucleotide metabolism, and calcium deposition (Sumner & Somers, 1943). It has been shown that as uterine weight, average embryonic weight, number of implantations, or number of viable embryos increases, there is also an increase in the total phosphatase activity of the uterus (Bredeck & Mayer, 1955). The vast amount of work on alkaline phosphatase indicates its probable role in glycogen synthesis and the transport of secretory substances across cell membranes (Wislocki & Dempsey, 1945; Dempsey &

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Wislocki, 1946; Pritchard, 1947; Moog, 1946; and Stafford *et al.*, 1947). The effect of estrogens and progesterones on these enzymes has been reviewed by Miner (1951), and Roberts & Szego (1953).

This study was initiated in order to determine (*a*) if regional differences in phosphatases exist in the uterus of the rat, and (*b*) if these differences correlate with embryo distribution patterns.

MATERIALS AND METHODS

Albino rats of the Wistar strain whose previous history was known were used in this study. The immature animals were 6-7 weeks old when killed. Virgin and primiparous rats were approximately 4-5 months old when killed. Vaginal smears were examined to select rats from different stages of the estrous cycle. All rats used were followed through two consecutive estrous cycles before being killed.

Pseudopregnant rats were obtained by placing an estrous female with a vasectomized male. If the animals had a vaginal plug and showed a diestrous type of smear the following morning when they were separated from the males, and continued to exhibit the same type of smear for 5 days, they were considered to be pseudopregnant. The females thus obtained were killed on the sixth day of pseudopregnancy; the first day being counted from the day prior to the finding of a diestrous type of smear.

Primiparous rats were obtained in the following manner: young virgin females were placed in breeding cages with mature males in the ratio of 5 to 1. From time to time the visibly pregnant females were separated and each one was kept in a separate cage. They were allowed to go to term. The number of young and the date of birth of the litters were recorded. The young were weaned at 23 to 24 days of age. After an additional 25 days each female was examined through two consecutive estrous cycles to check the uniformity of the cycles. The females having uniform cycles were killed at the required stage.

The rats were weighed before killing. As soon as each rat was stunned by a blow on the head, the abdomen was opened by a median incision. The reproductive tract was cut at the cervix and released from the surrounding tissue. It was then lifted and the uterine cornua were stretched in a manner which would produce as much uniformity along the length of each uterus and also between different uteri as possible. The uterus was pinned to a board covered with a cold wet paper towel. The mesometrium was then cut as close to the uterus as possible. The location of any placental scars along the uterine horns was recorded. Each uterine horn was then divided into three equal portions—the cranial, middle, and caudal segments. The segments were placed in separate glass, or rubber-stoppered, numbered vials and stored in ice. Mesometrial and antimesometrial halves were obtained by dividing the uterus approximately into two longitudinal halves. Each segment thus obtained was then weighed on a Roller–Smith torsion balance. Efforts were made to process each rat as

rapidly as possible to prevent loss of the enzymes. If the material was not used immediately, it was stored at or below –20° C.

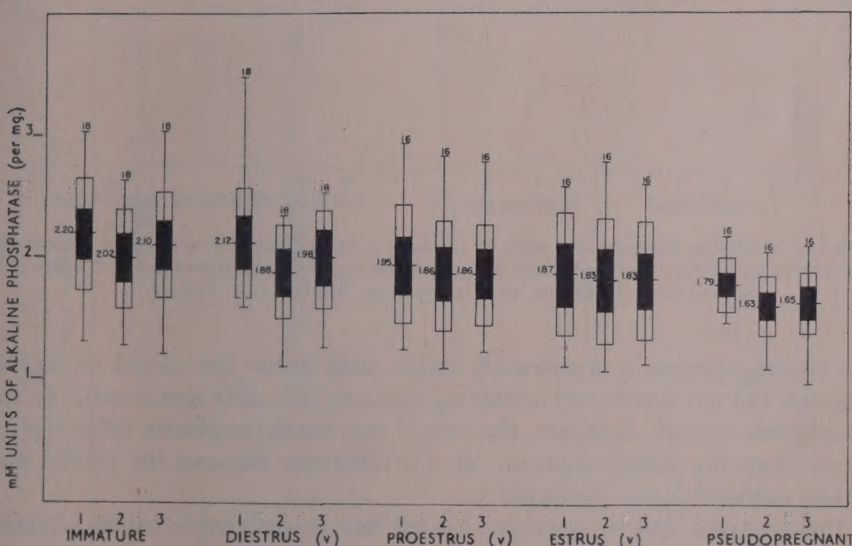
The phosphatases were determined by the ortho-nitrophenal phosphate method (Sigma Chemical Company, 1957). The uterine segments were homogenized in a Pyrex tissue grinder and the volume of the homogenate made to equal 2 c.c. Phosphatase activity was determined colorimetrically by a Bausch and Lomb 'Spectronic 20'. Phosphatase activity is reported in 'Sigma' units (Sigma Chemical Company, 1957).

Uterine phosphatase activity of the different segments was also estimated in two groups of estrogen- or progesterone-treated 30- to 32-day-old immature rats. One group was given each day for 6 days a subcutaneous injection of 0.1 c.c. of Mazola oil containing 0.55 mg. of estradiol benzoate per c.c. of oil. Another group was similarly treated with the same level of progesterone. A probability level of 0.05 or less was considered to be significant.

RESULTS

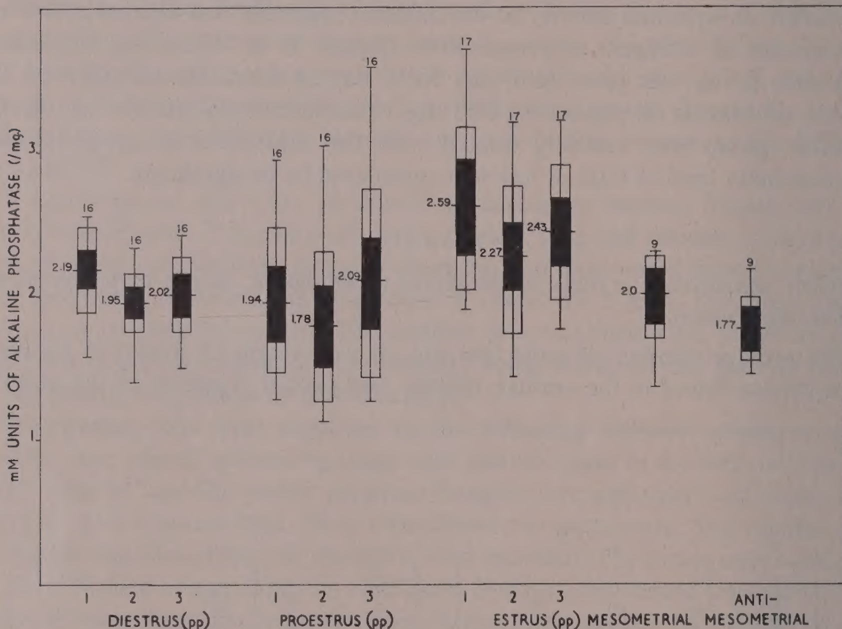
Alkaline phosphatase activity in the uterus of immature, virgin, pseudopregnant and uniparous rats

The average number of units (per mg. of wet weight of tissue) of alkaline phosphatase found in the cranial, middle, and caudal segments of the uterine



TEXT-FIG. 1. Alkaline phosphatase activity in segments of uterine horns from immature, virgin diestrous, virgin proestrous, virgin estrous, and pseudopregnant animals. The most anterior segment is indicated by 1, the middle by 2, and the most posterior by 3. One half of each black bar plus the white bar at either end denotes one standard deviation on either side of the mean. The white portion comprises two standard errors on either side of the mean. The middle vertical line shows the range with the figure at the upper end being the sample size.

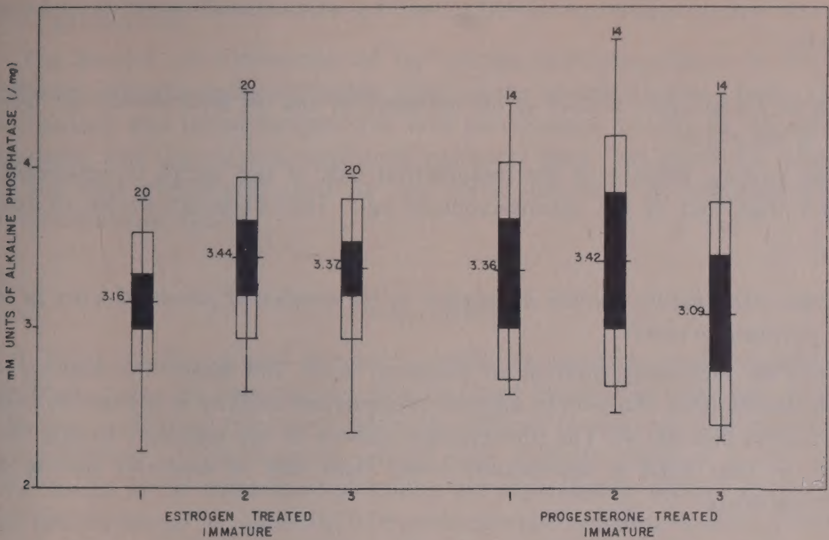
horns from the various groups of rats studied is indicated in Text-figs. 1 & 2. The significance of the disparity between the segments in the mean number of units of alkaline phosphatase activity was estimated by *t*-test (Fisher, 1950) within the immature, virgin, pseudopregnant, and postpartum groups. The virgin and postpartum groups were not subdivided into stages of the estrous cycle for this analysis. In the immature group there is no significant difference between any two segments. In the virgin and in the pseudopregnant animals,



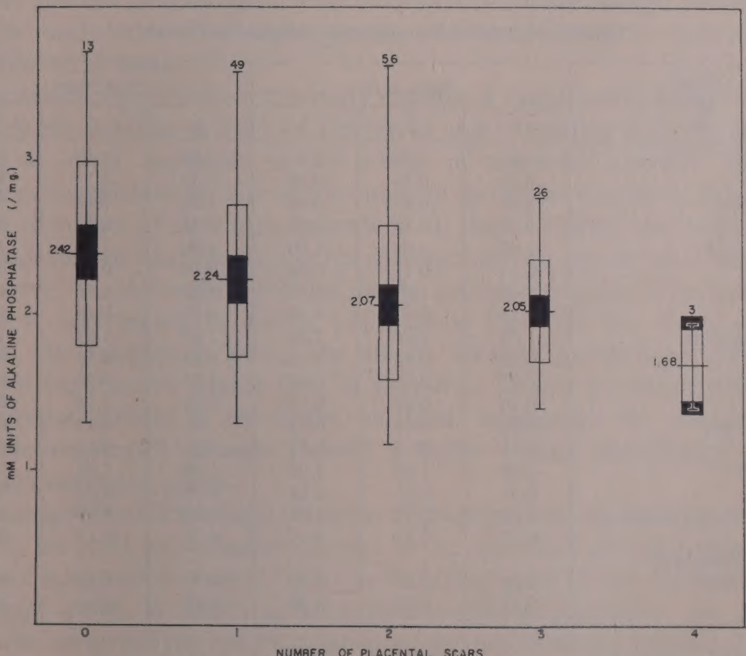
TEXT-FIG. 2. Alkaline phosphatase activity in segments of uterine horns from diestrous, proestrous, and estrous postpartum animals. Phosphatase activity for mesometrial and antimesometrial halves of virgin diestrous uterine horns is also given. Symbols as in Text-fig. 1.

the cranial segment is significantly higher than either the caudal or middle segment, but the middle and caudal segments do not differ significantly. In the postpartum animals, however, the cranial and caudal segments differ significantly from the middle segment, but the difference between the cranial and caudal segments is not significant.

The estrogen- and progesterone-treated rats, in addition to having a higher alkaline phosphatase activity in all the segments (Text-fig. 3), display a different pattern of alkaline phosphatase distribution. In these animals the middle segment has a higher phosphatase activity than either of the other two segments. The phosphatase in the cranial uterine segment from estrogen-treated animals is significantly lower than that of the other two segments, while the caudal



TEXT-FIG. 3. Alkaline phosphatase activity in segments of uterine horns from estrogen- and progesterone-treated immature animals. Symbols as in Text-fig. 1.



TEXT-FIG. 4. Alkaline phosphatase activity in segments of uterine horns grouped by numbers of placental scars in each segment. Symbols as in Text-fig. 1.

segment in the progesterone-treated animals has a significantly lower phosphatase activity.

Alkaline phosphatase activity of the mesometrial and the antimesometrial halves of the uterus

The enzyme activity of the mesometrial half of the uterus is significantly higher than that of the antimesometrial half. The values are shown in Text-fig. 2.

Alkaline phosphatase activity in relation to the number of placental scars in the postpartum uteri

With an increasing number of placental scars and hence the number of implantation sites, the level of alkaline phosphatase activity in the uterus tends to decline (Text-fig. 4). The phosphatase activity of the segments having two, three, or four scars is significantly lower than that of segments having no placental scars.

TABLE 1

Average units of acid phosphatase activity of the different segments of the uterine horns of the rat

Results are expressed in units mg. of tissue, wet weight

Groups with N	*Right horn*			*Left horn*		
	1	2	3	1	2	3
Immature	1.41	1.46	1.47	1.49	1.49	1.56
10	0.36	0.36	0.39	0.59	0.37	0.38
Diestrus (v)	0.90	0.95	0.98	1.04	0.94	0.96
9	0.11	0.15	0.13	0.26	0.17	0.09
Proestrus (v)	1.07	1.04	1.00	1.06	1.09	1.07
8	0.13	0.15	0.14	0.14	0.18	0.16
Estrus (v)	1.04	1.09	1.10	1.02	1.01	1.05
8	0.14	0.17	0.14	0.13	0.18	0.17
Pseudopregnant	1.05	1.04	1.02	1.07	1.09	1.13
8	0.19	0.16	0.19	0.21	0.12	0.11
Diestrus (pp)	0.92	0.92	0.95	0.92	0.99	0.98
8	0.16	0.19	0.16	0.16	0.14	0.19
Proestrus (pp)	0.96	0.95	0.93	1.01	0.95	0.93
8	0.11	0.16	0.13	0.11	0.14	0.16
Estrus (pp)	1.08	1.09	1.06	1.08	1.02	1.01
9	0.31	0.23	0.28	0.38	0.25	0.34
Estrogen-treated immature	1.02	0.95	1.08	0.94	0.95	1.09
10	0.25	0.17	0.19	0.22	0.17	0.16
Progesterone-treated immature	0.68	0.68	0.70	0.74	0.69	0.66
7	0.23	0.18	0.20	0.19	0.16	0.15

v, virgin; pp, postpartum.

The numbers 1, 2, and 3 at the top of the column of figures indicate cranial, middle, and caudal segments of the uterine horns. Standard deviations are listed beneath the means.

Acid phosphatase

The pattern of distribution of the uterine acid phosphatase is not very different between segments within most of the groups studied (Table 1). No correlation was found between the acid phosphatase activity in the different segments and the varying number of placental scars. No significant variation in the acid phosphatase activity was found between the mesometrial and antimesometrial halves.

DISCUSSION

Previous studies on the effects of circulating estrogens and progesterones on uterine alkaline phosphatase have involved chiefly histochemical methods. The results obtained from such studies are difficult to compare with quantitative measurements of total enzyme activity such as those obtained in this study. The results in the histochemical studies are expressed in relative reactivity of the specific tissues in the uterus of experimental and control animals. Pritchard (1949) has stated that estrogens cause a concentration of the enzyme in the uterine epithelium whereas progesterones cause it to disappear. Atkinson & Elftman (1947) and Hayashi *et al.* (1955) obtained essentially similar results in mice and rats respectively. On the basis of such studies it might be concluded that the total uterine alkaline phosphatase also would be highest when estrogens are high in the body.

The evidence presented in this study indicates a variable relationship between alkaline phosphatase activity of the uterus and circulating estrogen and progesterone levels, especially in the groups of untreated animals. The total alkaline phosphatase activity of the uterus in the immature rats is significantly higher than that of the virgin animals in all stages. Within the virgin group, the diestrous rats have the highest phosphatase activity, estrous the lowest, and, proestrous an intermediate activity. In the postpartum group all stages except proestrus are higher in uterine phosphatase than are the virgin animals. Within the postpartum group the uterine alkaline phosphatase activity at estrus is significantly higher than at proestrus. During pseudopregnancy the phosphatase activity is the lowest recorded. In contrast the estrogen- and progesterone-treated animals showed a higher uterine phosphatase activity than any untreated group.

It thus appears that the administration of exogenous estrogens or progesterone elevates the total phosphatase activity. In the untreated animal a much more complex relationship obtains. The second highest activity was recorded in the immature group in which presumably endogenous hormones are minimal while the lowest activity occurs in pseudopregnancy when the uterus is receiving hormonal stimulation.

A comparison of the various segments of the uterus in untreated and hormone-

treated animals also indicates a variation in the relationships between alkaline phosphatase and hormonal levels. At estrus, when oestrogens are high, the uterine alkaline phosphatase was found to be highest in the cranial segment in both virgin and primiparous animals. In the virgin animals the middle and caudal segments were almost equal in their phosphatase activity, but in the postpartum rats the middle segment had a lower activity than the caudal segment. In the estrogen-treated immature rats the cranial segment had the lowest phosphatase level while the middle segment had the highest.

The pseudopregnant rats which are assumed to have a high endogenous progesterone do not coincide with the progesterone-treated immature rats in the alkaline phosphatase distribution in the different segments of the uterine horns. In the pseudopregnant rats the cranial segment had the highest phosphatase level while in the progesterone-treated animals the middle segment had the highest. In the pseudopregnant rats the middle and caudal segments were almost equal in their phosphatase activity. The caudal segment had a lower phosphatase value in the progesterone-treated rats than either the cranial or the middle segment, both of which were almost equal. Thus, at those stages when estrogens and progesterones are probably high in the untreated animals, the alkaline phosphatase distribution in the different segments of the uterine horns is not at all similar to that of estrogen- and progesterone-treated immature rats. Perhaps the results are attributable to unphysiological doses of injected hormones.

The pattern of alkaline phosphatase activity in the uterine segments is seen to change after the first pregnancy. In the virgin animals the mean number of units of alkaline phosphatase activity of the middle and caudal segments does not differ significantly, but the cranial segment differs from both in its phosphatase activity. This is similar to the pattern of embryo distribution since the cephalic segment has the lowest number of embryos in the first pregnancy (Momberg & Conaway, 1956). In postpartum rats the cranial and caudal segments are similar, whereas the middle segment differs from both of these segments in its lower phosphatase distribution after the first pregnancy. This seems to coincide with the shift in the embryo implantation sites in the second pregnancy which results in greater numbers of implantations in the middle segment (Momberg & Conaway, 1956), suggesting a possible relationship between low alkaline phosphatase and the increased frequency in implantation. Such a relationship between the enzyme and implantation site is supported by the lower alkaline phosphatase activity found in the antimesometrial half of the uterus wherein implantation always takes place (Parkes, 1952). The uterine segments having a high number of placental scars show a low phosphatase activity. This inverse relationship between the phosphatase activity and the number of placental scars lends an added support to the suggestion that alkaline phosphatase may be related to embryo implantation.

SUMMARY

1. The uterine horns were divided into three regions (i.e. the cranial, middle, and caudal). Uteri from immature, virgin, pseudopregnant, primiparous, and from estrogen- and progesterone-treated immature rats were used in this study.

2. In the majority of the groups the cranial segment had the highest enzymatic activity, whereas the middle segment had lowest. The cranial and caudal segments of the rat uterus differed significantly in alkaline phosphatase activity in the virgin and pseudopregnant animals but not in the primiparous animals. Such a change in the phosphatase activity of segments after the first pregnancy seems to coincide with the reported anterior shift in implantation sites in the second pregnancy.

3. No significant variation was found in the concentration of acid phosphatase in the different segments that might correlate with embryo distribution patterns.

4. The phosphatase pattern in normal rats was not similar to that of immature rats treated with exogenous estrogen or progesterone. The dissimilarity may be due to unphysiological doses.

RÉSUMÉ

Activité phosphatasique alcaline dans l'utérus de Ratte

1. Les cornes utérines ont été divisées en trois régions (c'est-à-dire craniale, moyenne et caudale). On a utilisé dans ces recherches des utérus de rattes immatures, vierges, pseudo-gravides, primipares, et immatures traitées aux œstrogènes et à la progestérone.

2. Dans la majorité des groupes, le segment cranial possédait l'activité enzymatique la plus élevée, tandis que le segment moyen possédait la plus basse. Les segments cranial et caudal de l'utérus de ratte montraient une différence significative dans l'activité phosphatasique alcaline chez les animaux vierges et pseudogravides, mais pas chez les individus primipares. Une telle modification de l'activité phosphatasique des segments après la première grossesse semble coïncider avec le changement, signalé antérieurement, des points d'implantation lors de la seconde grossesse.

3. On n'a pas trouvé de variation significative dans la concentration en phosphatase acide des divers segments, qui pourrait être en corrélation avec le mode de répartition des embryons.

4. Le type phosphatasique des rattes normales était différent de celui de rattes immatures traitées aux œstrogènes exogènes ou à la progestérone. Cette dissemblance est peut-être due aux doses extra-physiologiques employées.

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An Analysis of the Postgastrula Differentiation of the Hypomere

II. The influence of endoderm and tissue mass in *Taricha torosa*

by CYRIL V. FINNEGAN[1]

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INTRODUCTION

THE initial report in this series (Finnegan, 1961) emphasized the role of the endoderm in the postgastrula differentiation of the hypomeric mesoderm in *Ambystoma punctatum*. The effect of the endoderm appeared to be modified when the mass of mesoderm involved was increased and, under the *in vitro* experimental conditions employed, the endoderm did not influence the splanchnic layer of the hypomere into new types of histogenesis (induction). Thus it was concluded that the endoderm aided the histogenesis of the splanchnic mesoderm in its vicinity in a synergistic manner rather than as an inductive tissue.

Further evidence of the mechanism of this assistance by the endoderm of the postgastrula development of the mesoderm has been obtained from similar *in vitro* studies with tissues from *Taricha torosa* neurulae. This report is concerned with results which substantiate the previously derived conclusions and, further, indicate at least one manner in which the endoderm effects its synergistic role.

EXPERIMENTAL PROCEDURES

T. torosa eggs were shipped air express from St. Mary's, California, and maintained in this laboratory in pond water at 7° C. I should like to note with gratitude my appreciation to Brother Lawrence Cory, F.S.C., St. Mary's College, for supplying eggs to this laboratory during the past several years. Explants were prepared as described in the earlier report on *A. punctatum* (Finnegan, 1961) and were cultured in modified Holtfreter or Niu–Twitty medium at 18° C. Briefly, the explants were prepared by excising a limited amount (approximately $0{\cdot}5 \times 0{\cdot}5$ mm.) of hypomeric mesoderm from the ventro-lateral flank of post-gastrula stages (stages 13–21; Twitty & Bodenstein stages—see Rugh, 1948) along with the overlying ectoderm. These explants quickly formed an ecto-dermal ball with the hypomere internal and constituted the hypomere only or (*M*) series. A second group of explants was prepared in a similar manner and a small mass of endoderm cells, taken from the lateral pharyngeal wall, was

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added to the ectodermal ball; these explants constitute the hypomere plus endoderm ($M+E$) series. The hypomere doubled ($2M$) series was prepared by combining two hypomere only (M) explants from similar-aged donors prior to the formation of ectodermal balls by these explants. A second method of increasing the amount of material from the mesoderm mantle was by increasing the length along the dorso-ventral axis. The anterior, posterior, and ventral borders of these explants were prepared as in the hypomere only (M) series (Finnegan, 1961) and the incisions were then continued dorsally through the intermediate mesoderm (mesomere) and into the somitic mesoderm (epimere), typically of somites 4–6. That portion of the somites lying immediately adjacent to the neural tube and notochord was excluded in order to delete known or suspected axial influences (see Yamada, 1950; Muchmore, 1951, 1958; Holtzer & Detwiler, 1953; Ebert, 1959). The ectodermal covering for these explants was restricted to that ectoderm lying ventral to a line approximately one-half way down the somite mesoderm, any additional ectoderm required to complete the covering being taken from the ventral-anterior area of the donor animal. These explants constituted the hypomere plus epimere ($M+Ep$) series.

The vitally stained explants were prepared by placing small pieces of cellophane previously stained with Nile blue sulphate (Rugh, 1948) on the anterior or posterior area of the explant immediately following its removal from the donor. The cellophane was held in place on the ectoderm so that the cells of the superficial epithelium were intensely stained in the small area of contact while the surrounding ectoderm cells were only lightly stained. Some penetration or diffusion of the stain to the more superficial mesoderm cells may have occurred during or following the short exposure of the explant to the cellophane. A similar procedure was used to produce superficial vital stain marks on the flanks of control embryos.

Explants were examined daily and sketches made of their development. At intervals during the culture period explants were fixed in Michaelis' fluid, sectioned at 8–10 μ and stained with haematoxylin and eosin for histological examination. The descriptions of histogenesis are based on the study of sections from approximately 50 per cent. of the explants in the larger groups, (M) and ($M+E$), and all of the explants in the two smaller groups, ($2M$) and ($M+Ep$).

EXPERIMENTAL RESULTS

Since, within an experimental series, the explants from all the postgastrula stages (i.e. stages 13–21) appeared to develop in a similar manner, they are discussed together, variation being noted where pertinent.

Hypomeric mesoderm (M) *series* (*66 cases*)

All the explants in this experimental series became vesicular in the first 2 to 4 days. Of the 17 cases with vital stain marks, those on which the mark was located anteriorly indicated a posteriad stretching of the stained cells by the

third to fifth day of culture and the posteriad migration of these superficial cells continued into the second week (Text-fig. 1). When the posterior area was stained the superficial cells did not migrate anteriad but the stain became less intense during the first week as some of the cells moved to the interior of the ball and then migrated anteriad. A small circular patch of mesoderm was present internally in the posterior-ventral area of these stained explants. In the unstained cases the ectoderm superficial to this patch appeared roughened and the slight external pigment characteristic of this species was more concentrated in the area. These observations are taken to be indicative of cell migrations similar to those demonstrated in the vital stained cases. At the end of the first week (control stage 36–39) or early in the second week (control stage 40) the mesoderm patch appeared to show a pink coloration in some cases but no further evidence of haematopoiesis was visible macroscopically. In the explants from the stage 20 donor group there occasionally appeared a second small internal mass of mesoderm cells in the dorso-posterior aspect of the ball (Text-fig. 1).

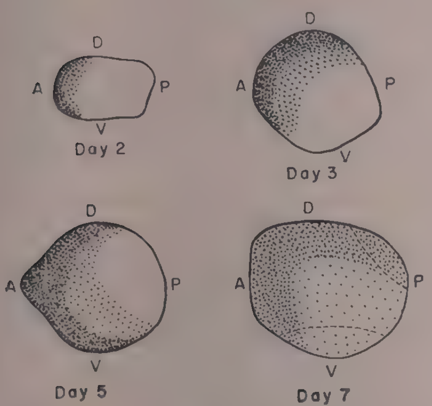


FIG. 1

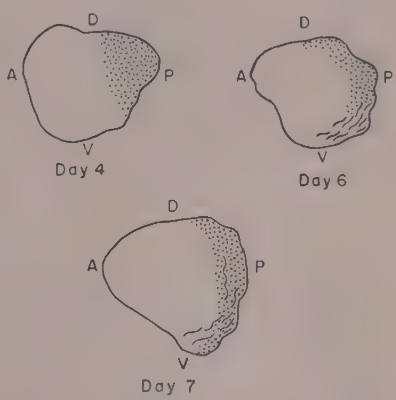


FIG. 2

TEXT-FIG. 1. Hypomeric mesoderm (*M*) series (stage 17 group). A vital stain-mark was placed on the superficial anterior end (*A*) of the explant and the cell displacement was sketched (at approximately × 30) during the first week of incubation. The dotted lines shown on day 7 indicate the typical ventro-posterior location of the mesoderm group found internally in all explants and the small dorsal mesoderm group occasionally found in the stage 20 explants.

TEXT-FIG. 2. Hypomeric mesoderm (*M*) series (stage 20 group). A vital stain-mark was placed on the posterior end (*P*) of the explant and the distribution of the stained cells sketched (at approximately × 30) during the first week of culture. No anteriad displacement of superficial cells took place. The wavy lines in the posterior area shown on days 6 and 7 have been superimposed on the sketches to indicate the location of the rough pigmented ectoderm observed in unstained explants.

These observations are indicative of a consistent migration of cells, and in control animals (stages 17–21) prepared with superficial vital stain-marks along the dorso-ventral axis at mid-trunk the displacement of cells (particularly noticeable following stage 24) indicated that the superficial flank tissue ventral to the yolk border elongated along the antero-posterior axis. Such results,

along with the confirming observations on the direction of ciliary beat (see Twitty & Bodenstein, 1941), demonstrate the maintenance of the original antero-posterior axis by the explanted tissues.

Examination of the sectioned material revealed, in the posteriorly located small mesoderm mass, haematoblasts and cells morphologically resembling erythrocytes but with unstained cytoplasm. Elsewhere the mesoderm appeared as a peritoneum underlying the epithelium and, in those explants fixed after 2 weeks of culture, the splanchnic mesoderm had differentiated as a second peritoneum lying internal to the somatic peritoneum. No granulopoiesis was evident though the stage 20 group produced small collections of acidophil cells and fibrous matrix in the splanchnic layer.

It could be seen that in none of these *T. torosa* explants did the degree of differentiation approach that obtained in the hypomere explants from stage 20 *A. punctatum* embryos (Finnegan, 1961) and thus 10 cases were prepared from *T. torosa* donors of stages 23–28. Both macroscopically and histologically these explants resembled those prepared from younger *T. torosa* developmental stages, though they did indicate the differentiation of endothelium and a larger number of acidophil cell groups in the splanchnic mesoderm, but no definite granulopoiesis could be identified. In general it appears that, under similar experimental conditions, the splanchnic mesoderm of *T. torosa* tends to disperse while that of *A. punctatum* more frequently remains as an internal mass, and the histogenesis observed reflects this difference.

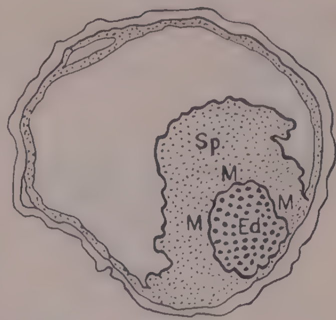
Hypomeric mesoderm plus endoderm ($M+E$) *series* (*47 cases*)

For the most part the behaviour of these explants in culture was similar to that observed in the mesoderm (M) series reported above. In a number of cases the endoderm resided in the ventral or anterior end of the ball accompanied by a group of mesoderm cells. Otherwise the endodermal group was located in the posterior end of the ball in the vicinity of the apparently enlarged posterior-ventral internal patch of mesoderm.

Histological examination showed the large collection of splanchnic cells in the vicinity of the endoderm to be continuous with the splanchnic peritoneum found elsewhere in the explant. Within the splanchnic mesodermal mass those cells located immediately adjacent to the endoderm retained their yolk content longer than those further removed and, in the stage 20 group after 2 weeks in culture, these latter cells of the splanchnic mass appeared to be differentiating a reticular tissue (acidophil cells and matrix) while those mesoderm cells more closely juxtaposed to the endoderm demonstrated mitotic figures and basiphil cytoplasm. In all cases, the explant areas distinct from the endodermal group were similar in histogenesis to the mesoderm (M) series (Text-fig. 3).

It seems then that the splanchnic mesoderm cells are retained in a group in the vicinity of the endoderm cells while elsewhere in the explant the dispersal encountered in the preceding (M) series occurs. The response of cytoplasm to

dye (basiphil or acidophil) in these cell masses might be taken, along with the position of the mitotic figures, to indicate that the more superficial splanchnic cells somewhat removed from the endoderm, but responding to the mesoderm mass, were undergoing histogenesis, while cells closely associated with the endoderm remained undifferentiated (Text-fig. 3). The production of a second peritoneum by the splanchnic mesoderm in the (*M*) series and in these (*M*+*E*) explants in areas removed from the endoderm would appear to be in agreement with the observations of Jacobson (1960) on *Taricha* and Nieuwkoop (1947) on *Triton* that removal of the endoderm from early embryos resulted in an increased formation of peritoneum from splanchnic mesoderm.



TEXT-FIG. 3. Hypomeric mesoderm plus endoderm (*M*+*E*) series (stage 20 group—two weeks). A composite figure made from photographs of several sections showing the endoderm (*Ed*) and the associated splanchnic mesoderm (*Sp*). Mitotic figures (*M*) were visible on three sides of the endoderm in one section and acidophil cytoplasm was observed in those cells of the mesoderm mass in the area surrounding the letters (*Sp*) in the illustration. ×160.

Hypomere doubled (2*M*) *series* (*7 cases*)

These few explants were prepared from late stage 17 and stage 20 donor groups and since the results were consistent they are included here. In the main, the histogenesis observed resembled that of the mesoderm (*M*) series and, though the stage 20 group produced a small endothelial area, the major splanchnic development continued to be peritoneum with a loose group of haematoblasts. Endoderm was added to two stage 20 explants and again there occurred the large collection of splanchnic mesoderm and the previously described histogenesis in the vicinity of these endoderm cells. Elsewhere these explants resembled the other (2*M*) cases and no evidence of axial organization such as had been observed in similar (2*M*) *punctatum* explants (Finnegan, 1961) was visible.

Hypomere plus epimere (*M*+*Ep*) *series* (*16 cases*)

Macroscopically, these explants from stage 17 and stage 20 donors resembled the (*M*) and the (*M*+*E*) series during the culture period in that they showed posteriad migration of the superficial material, an apparent involution of cells at the posterior end, and the appearance of two mesodermal groups within the vesicular ball (at the anterior and at the posterior-ventral ends of the original axis). At the end of the first week a duct-like structure could be seen coursing a short distance posteriad from the anterior mesoderm group, being particularly noticeable in several cases where vital stain had been placed anteriorly and the duct cells appeared as a short blue line along the wall of the ball (see discussion in Burns, 1955, of the origin of the pronephric duct from intermediate mesoderm

associated with somites 5–7 in *Ambystoma*). In the second week this duct increased in length; it reached the posterior mesoderm group in only one case.

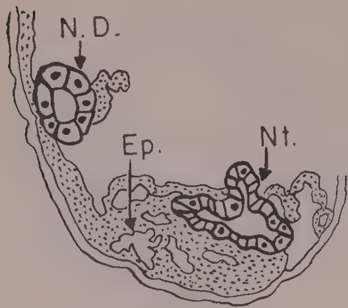


FIG. 4

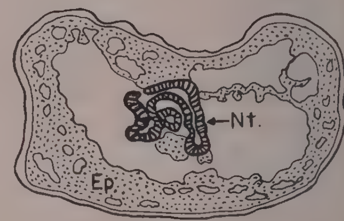


FIG. 5

TEXT-FIG. 4. Hypomeric mesoderm plus epimere ($M+Ep$) series (stage 20 group—10 days). A nephric duct (*N.D.*) has differentiated and in the associated epimere (*Ep.*) some attempt at the formation of a nephric tubule (*Nt.*) seems to have occurred. ×160.

TEXT-FIG. 5. Epimeric mesoderm from a stage 17 *T. torosa* donor cultured in an ectodermal ball for 16 days. A well-differentiated nephric tubule (*Nt.*) was produced on the edge of a mass of epimere cells (located one section prior to illustrated area). ×160.

Histological examination showed that the anteriorly located group of mesoderm cells initially appeared as a loose collection of cells (parenchyma) which became aligned into columns of acidophil cells in the second week of culture and only in the stage 20 group produced a nephric tubule or two (Text-fig. 4). The hypomere lying ventral to the nephric duct appeared to remain as a more substantial group of basiphil cells than elsewhere in the explant and a few fibroblasts developed in the second week. Further posteriad the histogenesis resembled that of the other experimental series; no differentiating mesoderm group could be found in either the epimere or the hypomere, other than the possible haematopoietic cells, indicating that cell dispersal had occurred.

While myogenesis had not been anticipated (Muchmore, 1951, 1957) and was not observed, the failure of these ($M+Ep$) explants to produce more in the way of nephric tubule differentiation is interesting and puzzling when contrasted with results obtained in this laboratory in experiments in which the same area of epimere from stage 17 donors was cultured alone in ectodermal balls for over 2 weeks and in which well-developed tubules were produced (Text-fig. 5).

DISCUSSION

In an earlier publication (Finnegan, 1953) observations were reported of the endodermal effect on the behaviour of hypomere when the two tissues were placed in a confined space in a hanging drop culture. At that time it was stated (p. 379) that ‘the migration of the mesodermal elements of these explants was

retarded considerably and it appeared that the tissue affinity described by Holtfreter for these two tissue layers of the embryo was manifested in the inhibition of the migration commonly associated with mesodermal explants'. Similarly, when ectoderm, mesoderm, and endoderm were combined and cultured under the same experimental conditions (p. 381), 'the typical mesodermal migration took place from the ectodermal region of the explant' but 'the majority of the mesodermal cells entering the area between the ectoderm and endoderm rounded up and, though not physically blocked from continued movement away from the explant, ceased migration'.

The experimental results reported here demonstrate that, in an ectodermal ball, *Taricha torosa* hypomere taken from postgastrula stages behaves in a similar manner, that is, it spreads in the absence of endoderm but remains as a mass of cells in close association with added endoderm. In this manner the endoderm would aid the subsequent differentiation of the hypomere since within the retained mass the 'localized predispositions for differentiation' (Muchmore, 1951) which are 'system-dependent' (Grobstein, 1959) may exert their influence. Muchmore (1957, 1958; see also Ebert, 1959) has postulated such a role for the endoderm and other neighbouring tissues in preventing the migration of somitic mesoderm cells and thereby aiding their subsequent differentiation.

A further role of the endoderm is also indicated by these results. Initially, the endoderm acted to retain the mesoderm in its vicinity and, after this relationship had existed for a time (by late in the second week of culture), the splanchnic cells juxtaposed to the endoderm were observed to be engaged in mitotic activity, as though they were part of a germinal layer. It must be made clear that those splanchnic mesoderm cells within the mass but removed from the immediate vicinity of the endoderm were differentiating while those cells directly in contact with the endoderm appeared to remain undifferentiated. Again, as in previous observations on *T. torosa* tissue (Finnegan, 1953), it was difficult to escape the impression that the endoderm group, in addition to discouraging migration, was also maintaining the mesoderm cells on its immediate periphery in a mitotically active, undifferentiated state. Possibly in this role the endoderm is the heterogeneous tissue, contact with which in some manner releases the mesoderm cells from the mitotic inhibition of adjacent mesoderm cells, as suggested by Weiss (1959, pp. 90–91), or its role may be more specific in this case. In either event it is to be noticed that the initial activity of the endoderm in retaining a mesodermal mass favours the differentiation of the mesoderm cells, while it is only later that the endodermal effect on mitosis can be observed in those mesoderm cells of the mass which are in contact with the endoderm cells. A similar conclusion was made from the analysis of mitotic counts in the *A. punctatum* explants (Finnegan, 1961) in which it was shown that the endoderm initially effected differentiation and later, in the third week of culture, seemed to influence mitotic activity. In comparing the results on the two species, it would appear that

the discouragement of splanchnic mesoderm dispersal occurs earlier in *A. punctatum* than in *T. torosa*.

The results obtained in the experimental series in which hypomere and epimere mesoderm (*M+Ep*) were included in the ectodermal ball indicate that the stimulus to nephric differentiation also acts (*in vitro*) to retain the neighbouring mesoderm as cell groups by preventing their dispersal. Perhaps it is that while cells are migratory any inductive tissue must first act (with specific cell adhesions) in such a manner as to obtain quiescence in the responding tissue and that a morphogenetic field initially is composed of the cells which have so responded and are so retained.

SUMMARY

1. A small mass of *T. torosa* postgastrula (stages 14–21) hypomere with its associated ectoderm, when cultured *in vitro*, demonstrates cell displacement posteriad along the original antero-posterior axis.

2. Histological examination of these explants indicates that the splanchnic mesoderm disperses and differentiates, as a peritoneum for the most part, with a loose group of haematoblasts residing in the cavity formed.

3. When a small group of endoderm cells was added to the hypomere-ectoderm ball, the results were similar to the above except that splanchnic mesoderm in the vicinity of the endoderm remained as a cell mass rather than dispersing as mesothelium. Late in the second week of culture mitotic figures became more prevalent in the splanchnic mesoderm in the immediate vicinity of the endoderm than elsewhere in the splanchnic cell mass.

4. When epimere (somite) mesoderm was included in the hypomere-ectoderm ball, a nephric duct developed anteriorly and mesoderm cells remained as a cell mass in the vicinity of this duct.

5. The results are discussed as indicating that the endoderm retains a mesoderm mass in its vicinity and thereby assists the differentiation of this mesoderm.

RÉSUMÉ

Analyse de la différenciation de l'hypomère après la gastrulation

II. L'influence de l'endoderme et de la quantité de tissu chez *taricha torosa*

1. Une petite quantité d'hypomère de *Taricha torosa* après la gastrulation (stade 14–21), avec son ectoderme associé, montre, quand elle est cultivée *in vitro*, un déplacement de cellules vers l'arrière, le long de l'axe antéro-postérieur initial.

2. L'examen histologique de ces explants montre que le mésoderme splanchnique se disperse et se différencie principalement en péritoine, avec un groupe résiduel d'hématoblastes qui prennent place dans la cavité formée.

3. Lorsqu'un petit groupe de cellules endodermiques est ajouté à la sphérule formée d'hypomère et d'ectoderme, les résultats sont les mêmes que ci-dessus,

à part le fait que le mésoderme splanchnique, au voisinage de l'endoderme, constitue une masse de cellules au lieu de se répandre en un mésothelium. Plus tard, dans la deuxième semaine de culture, les figures mitotiques deviennent plus fréquentes dans le mésoderme splanchnique au voisinage immédiat de l'endoderme qu'aux autres niveaux de la masse cellulaire splanchnique.

4. Lorsque du mésoderme d'épimère (somite) est inclus dans la sphérule formée d'hypomère et d'ectoderme, un tube néphritique se développe vers l'avant, et les cellules mésodermiques restent amassées au voisinage de ce tube.

5. La discussion des résultats porte sur l'indication que l'endoderme retient du matériel mésodermique dans son voisinage et de cette façon joue un rôle dans la différenciation de ce mésoderme.

ACKNOWLEDGEMENT

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(*Manuscript received 19: xii: 60*)

Experiments on the Maternal-Foetal Barrier in the Mouse

I. A Test for the Transmission of Maternal Erythrocytes across the Mouse Placenta following X-irradiation

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INTRODUCTION

EXPOSURE of new-born mammals and birds to foreign cells can induce a specific immunological non-reactivity ('tolerance') towards grafts of donor tissues later in life. In the case of mammals, if large numbers of maternal cells were to leak into the foetal circulation during pregnancy, one possible immunological consequence would be that the offspring would be tolerant of the mother's tissues in later life. In the human species there is evidence that leakage of maternal cells can occur on a sufficient scale to induce immunological tolerance of rhesus blood-group antigens, detectable in some rhesus-negative offspring of rhesus-positive mothers (Owen, 1957). In the mouse, however, Billingham, Brent, & Medawar (1956) cite the results of skin-grafting from mother to young as evidence that spontaneous leakage occurs very rarely, if at all.

Mitchison (1953) showed in mice, and Woodruff (1957) in rats and rabbits, that the experimental induction of a state of immunity in the pregnant female against her own young is without detectable effect upon the latter's survival. A reasonable inference is that, at least in the case of immunologically competent cells, massive mother-to-foetus transmission does not occur.

Lengerová (1957) was able to produce tolerance in rats by irradiating the mother's uterus with 200 r. on day 15 of pregnancy. The maternal cells responsible for inducing tolerance in this case might be derived from the maternal component of the damaged placenta itself, or alternatively they might be circulating cells passing into the foetus through placental defects caused by the treatment.

Moulton, Stimpfling, & Storer (1960) attempted, without success, to repeat

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Lengerová's result using mice. They did not, however, ascertain whether tolerance could be induced by orthodox procedures in the donor-recipient combination which they used. It is therefore not easy to evaluate the results of their experiment.

If irradiation damages the placenta so that cells pass from the maternal into the foetal circulation, the leak should be demonstrable by injecting labelled cells into the mother's blood and recovering them in the blood of the young. In the work to be described, mice were exposed to X-rays in varying doses at varying times during pregnancy (see below). Whole blood labelled with radioactive chromium was transfused into pregnant animals within 2 days of their irradiation. Under the conditions employed, the overwhelming majority of the chromium is fixed by the erythrocytes in the inoculum (Mollison & Veall, 1955). The radioactivity of maternal blood, of whole new-borns, and of the blood of certain new-borns was compared on the day of delivery.

MATERIALS AND METHODS

Pregnant mice from a random-bred stock were irradiated at the times and doses stated below. Only the abdomen was exposed to the beam, the remainder of the body being shielded by $\frac{3}{32}$ of an inch of lead. Röntgen values shown are calculated at the anterior abdominal wall and include the expected contribution of back-scatter. A Westinghouse radiotherapy unit was operated at 230 kV., 15 mA., at a distance of 25 cm., with 0·5 mm. Cu and 1 mm. A1 filters, giving a half-value layer of 1·2 mm. Cu.

In most cases 5·4–8·0 μc. of radiochromium activity were delivered either intravenously or intraperitoneally in 0·15–0·20 ml. of whole blood. For labelling, 4 volumes of whole blood were incubated for 1 hour with 1 volume of an isotonic solution of sodium chromate containing 27 μg. Cr, of approximately 200 μc. activity, per ml. Judged by the activity of subsequent blood samples, intraperitoneal injection was about one-third as effective as intravenous injection. In a few cases 0·2 ml. of a solution of $Na_2{}^{51}CrO_4$, having 8–10 μc. activity, was injected directly into a vein, but blood samples proved to be weakly labelled by this method.

At all dose levels above 400 r., mice were given 2·5 mg. of progesterone in oil subcutaneously on day 18 to ensure maintenance of pregnancy until the following day, permitting (*a*) the anticipated effects of radiation on the placenta to operate for a standard time, and (*b*) the offspring to be delivered by Caesarian section so that the uterus could be examined for dead foetuses.

Radioactivity was measured in a Panax well-type scintillation counter at settings of 1,000 E.H.T. volts, 10 discriminator volts, and 100 seconds. Counts shown have been corrected for background radiation. Whole new-borns, or blood samples, were placed in the bottom of a test-tube within the well of the counter. Blood samples ranged from 0·040 to 0·065 ml., but were equal for each mother and her offspring. Combination of blood from two new-borns was

sometimes necessary to make 0·04 ml. Blood was collected from adults by tapping the retro-orbital venous plexus with a thick-walled capillary pipette, and from new-born mice by decapitation.

RESULTS

Twenty-nine pregnant mice were irradiated in single doses which ranged from 184 to 1,000 r. Two hundred and five live and 74 dead young were counted in all. The general outline of the experiment is shown in Table 1.

TABLE 1

Outline of the experiment

Day of pregnancy	*Number of pregnant mice*	*Dose-range (röntgens)*
13	4	200–480 (foetal abnormalities and deaths above 300 r.)
14	18	200–1,000 (foetal abnormalities and deaths above 700 r.)
15	4	184–400
16	1	184
17	2	184–400

An unexpected observation was that almost all young, when counted as whole animals, showed weak activity, ranging up to 1,300/100 sec. with an average of 355.

However, when the bloods of the seemingly most active young were counted, no significant activity was present, with two exceptions to be described below. The source of the radioactivity found has not been determined, although skin, stomach with milk content, liver, and spleen have been found to be inactive.

Two exceptional results occurred with mice receiving 400 r. on day 13 and day 15. In the latter case, the maternal blood gave 6,041/100 sec. counts, one of seven whole new-borns gave 5,994, and its blood 2,657. The counts of the other six live whole new-borns in that litter ranged from 343 to 526, and the blood of one of them gave 16 counts. No activity was detected among the six new-borns of another litter treated with 400 r. on day 15, or in any of the offspring of mothers receiving the same or smaller doses on days 16 and 17 of pregnancy.

The mouse which was treated on day 13 was unfortunately poorly labelled and her blood on day 19 gave only 500/100 sec. counts. Two members of the litter of 14 were alive, and their pooled blood gave 148 counts. Since the standard error of this figure is 37, the excess of activity over background must be accepted as real. But because of the unsatisfactory labelling of the mother's blood, and the negative results obtained with higher radiation doses administered on the same and following day of pregnancy, we are hesitant to regard it as an effect of the treatment. We prefer to ascribe both of these cases to spontaneous leakage of maternal cells into the foetal blood. On this basis we may estimate from our

data that the frequency of this as a natural phenomenon is in the region of 1 per cent. of foetuses.

DISCUSSION

The idea that, in the mouse, the placental barrier to circulating erythrocytes can be breached by X-irradiation is not supported by our results. It also appears that spontaneous breakdown of the barrier sometimes occurs, but only with extreme rarity. This presents a striking contrast with the situation in the human species, where the transmission of erythrocytes from mother to foetus is relatively common. Many investigators, including Hedenstedt & Naeslund (1946), Naeslund & Nylin (1946), Naeslund (1951), Mengert *et al.* (1955), and Macris, Hellman, & Watson (1958), have been able to recover maternal erythrocytes labelled in various ways from the blood of the new-born infant. The estimates of frequency given by these authors show considerable heterogeneity, but by pooling their figures we can arrive at an estimate which approaches 50 per cent. of all infants tested. This may be contrasted with the estimate of about 1 per cent. of foetuses tested in our material.

The degree of mixing found in the two positive cases can be estimated as 44 per cent. and 30 per cent. respectively. These values are surprisingly high. On the other hand, they fall near the centre of the range of values found in human material by the authors previously cited. Even if all estimates based on the use of radioactive tracers are ruled out of court as being conceivably subject to difficulties of interpretation, we are still left with the results obtained by Macris *et al.* who exploited hereditary sickle trait as an erythrocyte marker. Two of their three positive cases betrayed a degree of admixture in the region of 20 per cent., confirming that transplacental leakage, when it occurs, is by no means always a minor or marginal phenomenon.

SUMMARY

Leakage of chromium-labelled erythrocytes from the circulation of pregnant mice into the foetal circulation could not be detected, either following X-irradiation at various dose-levels and gestational stages or in untreated pregnancies. Two exceptions were encountered out of a total of 279 foetuses examined. This was taken as an estimate of the spontaneous incidence of transplacental leakage of maternal erythrocytes and contrasted with the relatively high incidence encountered in the human species.

RÉSUMÉ

Expériences sur la barrière placentaire chez la souris

I. *Test pour la transmission des érythrocytes maternels à travers le placenta de la souris, après irradiation aux rayons X*

Le passage d'érythrocytes marqués au chrome, de la circulation de souris gestantes vers la circulation fœtale n'a pu être détecté, d'une part après

irradiation par des doses variées de rayons X et à différents stades de la gestation, d'autre part chez des souris non traitées. Deux exceptions ont été enregistrées sur un total de 279 fœtus examinés. Ce résultat est considéré comme une évaluation de l'incidence spontanée du passage transplacentaire des érythrocytes maternels et contraste avec l'incidence relativement élevée enregistrée dans l'espèce humaine.

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Experiments on the Maternal-Foetal Barrier in the Mouse

II. A Test for the Transmission of Maternal Serum Albumin into the Foetal Circulation following X-irradiation

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INTRODUCTION

LENGEROVÁ (1957) obtained indirect evidence that placental permeability to transplantation antigens could be induced in the rat by delivering 200 r. of X-irradiation to the pregnant uterus on the 15th day of gestation. The young were found subsequently to be immunologically tolerant of grafts of maternal skin. The passage of transplantation antigens into the foetus in sufficient quantity to induce tolerance strongly suggests transplacental leakage of leucocytes (since erythrocytes are ineffective in inducing tolerance to grafted skin).

Finegold & Michie (1961) made a direct test for mother-to-foetus transmission of labelled erythrocytes in the mouse following X-irradiation at various stages of pregnancy, but obtained negative results. In order to characterize further the limits to permeation from mother to foetus, we have tested for the transmission, both with and without X-ray treatment, of albumin-bound Evans blue and of any free dye which may, under the conditions employed, be present in the circulation of the mother.

MATERIALS AND METHODS

Pregnant mice were exposed to X-rays at a dose of 300 r. Evans blue (T-1824) in standard concentration was injected into irradiated and non-irradiated females on day 19 of pregnancy. After a half-hour interval the transmission density of the maternal and foetal blood was compared.

The experimental mice were female F_1 hybrids between the C57BL and A inbred strains. Pregnancies were obtained by mating them with males of the same genetic constitution as themselves. The dates of mating were established by daily examination of the females for copulation plugs. For a given test, two

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females were used at the same stage of pregnancy, one receiving the X-ray treatment and the other serving as control. In each case both mice were injected subcutaneously with 2·5 mg. progesterone ('Lutocyclin', CIBA Laboratories) on day 17 and day 18 of pregnancy, counting the day on which the copulation plug was found as day 0. This was done to ensure maintenance of pregnancy until day 19, on which the measurements on maternal and foetal blood were made. The full-term foetuses were delivered on day 19 by Caesarian section, since new-born mice can ingest the dye from the mother's milk.

The pregnant females received whole-body X-irradiation at various stages of gestation ranging from day 13 to day 19. A Westinghouse radiotherapy unit was operated at 230 kV. 15 mA., at a tube-to-floor distance of 50 cm. At this distance the field diameter is 8·0 cm., and the effective radiation dose, allowing for back-scatter, 150 r./min.

Typically, injections of dye were made into the tail vein, the standard dose being 0·3 ml. of a 2 per cent. aqueous solution of Evans blue. All solutions were filtered before use.

Blood samples were taken from the adult females with a specially calibrated pipette previously flushed through with heparin ('Liquemin' Roche), and from foetuses with the same pipette following decapitation. The blood of the females was taken in most cases from the retro-orbital venous plexus, but in some cases from the abdominal aorta immediately post mortem. In many cases blood from two or three foetuses was pooled to make up the sample for measurement. All samples were of 0·05 ml. volume, immediately diluted in 4·95 ml. of 3·8 per cent. sodium citrate. After centrifugation at 2,000 rev./min. to remove the erythrocytes, the supernatant was taken for spectrophotometric examination.

The transmission density of the samples of diluted plasma obtained as described above was determined with a Unicam spectrophotometer operated at a wavelength of 6250 Å. This wavelength was selected as giving a favourable discrimination between Evans blue and any traces of haemoglobin which might be present as contaminant.

RESULTS

Thirteen pregnant mice were examined of which seven were irradiated in a single standard dose of 300 r. Except in the case of day 15, every irradiated mouse had a non-irradiated control.

Blood samples were taken from 97 foetuses in all, 55 coming from the irradiated mothers and 42 from the control mothers.

A summary of the results obtained is shown in Table 1.

In none of the young examined could any significant increase in transmission density be found, although a satisfactory high level of dye was obtained in every female injected. The median estimate for new-born blood was 17 μg./l. in the offspring of irradiated females and 23 μg./l. in the control young. These values

are scarcely distinguishable from experimental and instrument error, having in mind that the average concentration in the blood of the mothers was 3,501 μg./l. and 3,400 μg./l. respectively.

TABLE 1

Summary of results

Numbers in brackets denote the number of foetuses examined. The number following the < sign in each case represents the highest reading obtained in the litter

Day of pregnancy on which irradiation was administered	*Treated mice*			*Control mice*		
	Concentration of dye (μg./l.) in			*Concentration of dye (μg./l.) in*		
	Maternal blood		*Foetal blood*	*Maternal blood*		*Foetal blood*
	At start of ½-hour period	*At end of ½-hour period*	*At end of ½-hour period*	*At start of ½-hour period*	*At end of ½-hour period*	*At end of ½-hour period*
13	4,308	3,846	< 31 (8)	4,231	3,708	< 62 (8)
14	4,692	3,923	< 31 (9)	4,369	3,831	< 31 (10)
15	4,138	3,969	< 15 (10)	—	—	—
16	4,277	4,000	< 15 (9)	4,123	3,785	< 46 (8)
17	*	3,723	< 31 (5)	*	3,000	< 46 (5)
18	3,538	3,277	< 62 (6)	4,123	3,538	< 92 (5)
19	2,646	1,769	< 46 (8)	3,985	2,538	< 62 (6)

* Dye injected intraperitoneally: see text.

In the case of day 17 of pregnancy the intravenous injection was unsuccessful in both the irradiated and control mothers and an intraperitoneal injection of 0·6 ml. of the 2 per cent. solution of Evans blue had to be given. A 3-hour period was given (instead of the usual half-hour as in the others) for the dye to be absorbed into the blood from the peritoneal cavity. The blood sample taken at Caesarian section showed that a satisfactory level of Evans blue in the blood was obtained (see Table 1). On removal of the foetuses it was observed that the peritoneal cavity including the uterus and its appendages was deeply stained with dye. In spite of this, however, no trace of dye could be detected in the blood of the foetuses.

DISCUSSION

Our results strongly suggest that serum albumin does not cross the mouse placenta or associated membranes. An alternative possibility must, however, be accorded formal recognition, namely dissociation of the dye-protein complex in the placenta, with passage of the larger molecule but not the smaller.

Opposing views are held concerning the permeability of the placenta to various substances in various species. Hagerman & Villee (1960) state that the discrepancies are due to the different dosages of the test substance given, the different species of animals and their respective types of placenta, and to the difficulties of chemical or physiological measurement.

The transfer of serum-proteins from maternal to foetal blood has been

studied in many animals including man. In some cases the evidence for transmission to the foetus has been based on immunological studies comparing the titre of specific antibodies in maternal blood with their titre in foetal or cord blood. Hagerman & Villee, however, still entertain some doubt as to whether these antibodies (mostly gamma-globulins) cross the placenta at all.

The work of Brambell and his school (Brambell, Hemmings, & Rowlands, 1947; Brambell, Hemmings, Henderson, & Oakley, 1952; Brambell, 1954; Brambell, Halliday, Brierley, & Hemmings, 1954; Hemmings & Oakley, 1957) indicates that the yolk-sac splanchnopleur rather than the placenta is the major pathway of antibody transfer to the rat and rabbit embryo. A selection according to the character of the protein occurs during this process. Brambell, Halliday, Brierley, & Hemmings (1954) state that there is no experimental evidence that maternal antibodies cross the human placenta directly. Brown, McGandy, Gillie, & Doyle (1959), however, do not support this generalization.

There is also some disagreement with regard to the permeability of the placenta to other proteins.

Whipple, Hill, Terry, Lucas, & Yuile (1955), studying the transfer of iodine-labelled serum albumin in rabbits and dogs, found labelled albumin in the rabbit foetuses but not in the dog foetuses. On the other hand, Shmerling's experiments cited by Hagerman & Villee (1960) with ^{14}C-labelled proteins in rats detected no transfer across the placenta. The same method was also used by Whipple and his colleagues with the same negative results.

Bangham, Hobbs, & Terry (1958) in their experiments on transfer of serum-proteins in the rhesus monkey demonstrated the direct transmission of homologous maternal albumin and gamma-globulin to the foetal circulation. They concluded that it was selective, in that gamma-globulin was transmitted 15–20 times more easily than albumin. They also believe that the transfer is not via the amniotic fluid but is transplacental.

The present results indicate that mice resemble rats and dogs rather than monkeys and rabbits in respect of mother-to-foetus transmission of serum albumin. It should be noted that they exclude not only the placental pathway of transmission but also the alternative pathway via the uterine lumen and yolk-sac splanchnopleur implicated by the above-mentioned studies of Brambell and his colleagues on the transmission of γ-globulin.

Since only a short period at full term was allowed for transplacental transmission of the label, the results have decisive application only to the terminal stage of pregnancy. At this terminal stage it can be concluded, subject to the formal qualification stated at the outset of the discussion, that mouse albumin, which has a molecular weight of 70,000, is unable to pass. If substantial quantities of free dye were also present in the maternal blood, unattached to albumin, then the upper limit which our results set to permeability would be greatly lowered, since Evans blue has a molecular weight of 961 and, in our experience, will not pass through a dialysis membrane of 25 Å pore diameter. We have not

undertaken the rather complex studies which would be needed to settle this question.

SUMMARY

Serum albumin labelled with Evans blue did not pass from the maternal into the foetal circulation in 13 pregnant mice, of which 7 had received 300 r. X-irradiation at stages ranging from day 13 to day 19, and 6 were untreated. The bearing of this result on studies of placental permeability in other species is considered.

RÉSUMÉ

Expériences sur la barrière placentaire chez la souris

II. *Test pour la transmission du sérum albumin maternel dans la circulation fœtale, après irradiation aux rayons X*

Le sérum albumin marqué au bleu Evans ne passe pas de la circulation maternelle dans la circulation fœtale chez 13 souris gestantes: 7 avaient reçu 300 r à des stades allant du 13e au 19e jour, et 6 n'avaient pas été traitées. La portée de ces résultats sur l'étude de la perméabilité placentaire chez d'autres espèces est discutée.

ACKNOWLEDGEMENTS

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L'Influence de divers mésenchymes sur la différenciation de l'épithélium pulmonaire de l'embryon de Poulet en culture *in vitro*

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AVEC DEUX PLANCHES

Le poumon embryonnaire de Poulet prélevé à 5 jours d'incubation offre l'aspect d'un petit sac mésenchymateux enveloppant un conduit épithélial qui présente deux ou trois dilatations. Les deux ébauches entrent en contact l'une avec l'autre pour édifier l'organe.

Nous nous proposons de chercher comment se produit cette différenciation. Nous nous sommes posé trois questions:

1) Que devient le conduit épithélial isolé du mésenchyme pulmonaire? Ce dernier est-il indispensable à l'édification du poumon?

2) Un poumon peut-il se développer à partir d'épithélium et de mésenchyme pulmonaires préalablement dissociés puis réassociés?

3) Un mésenchyme hétérologue induit-il l'épithélium à se ramifier et à entrer dans la constitution de l'organe étranger? Ou au contraire, l'épithélium pulmonaire, quel que soit le mésenchyme qui lui est associé, édifie-t-il toujours un système de ramifications de type pulmonaire?

MATÉRIEL ET MÉTHODES

Nous avons employé une méthode comparable à celle qu'a utilisée Grobstein (1953, 1955) pour l'étude de l'interaction entre l'épithélium et le mésenchyme du métanéphros et de la glande salivaire de Souris au cours du développement.

Le poumon, prélevé à 5 jours d'incubation, est plongé dans une solution de versène à 1‰, puis dans une solution de trypsine à 1‰. Une dissociation mécanique d'abord à la pipette, ensuite à l'aide de pinces et de scalpels fins, permet de séparer l'épithélium du mésenchyme qui l'entoure.

Nous déposons cet épithélium sur un fragment de membrane vitelline étalée sur le milieu de culture (Wolff, 1960). Cette membrane favorise l'étalement des explants et permet une meilleure culture. Nous utilisons le milieu de culture

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standard de notre laboratoire (Wolff & Haffen, 1952) additionné de sérum. Le milieu a donc la composition suivante:

Gélose à 1 pour cent dans du liquide de Gey	6 gouttes
Extrait embryonnaire dilué de moitié dans du liquide de Tyrode[1]	3 ,,
Liquide de Tyrode	1 ,,
Sérum de Poulain	3 ,,

L'épithélium est associé à du mésenchyme homologue ou hétérologue. Le résultat des associations est acquis après 4 à 5 jours de culture. Les explants sont fixés au liquide de Bouin entre le quatrième et le sixième jour de culture. Les uns sont coupés et colorés à l'Hémalun-Éosine: le contrôle histologique nous renseigne sur la structure de l'épithélium et l'importance des ramifications. Nous colorons les autres explants *in toto* au Carmin chlorhydrique alcoolique pour observer leur structure morphologique d'ensemble.

Dans les expériences d'associations hétérologues, nous avons cultivé des témoins de différentes catégories: *a*) le poumon controlatéral entier; *b*) l'épithélium isolé; et *c*) l'épithélium réassocié à du mésenchyme pulmonaire.

RÉSULTATS

Origine et développement du poumon embryonnaire de Poulet

Au stade de 23 somites, la portion respiratoire ventrale du pharynx est séparée par une petite constriction de la portion branchiale dorsale. Elle forme rapidement un étroit canal et pousse dans sa partie postérieure deux excroissances: ce sont les ébauches des poumons et des sacs aériens. Les bourgeons pulmonaires sont donc pairs dès leur début.

A 5 jours d'incubation, l'un des poumons se présente comme un sac mésenchymateux enveloppant un petit conduit épithélial. C'est à ce stade que l'épithélium commence à ébaucher les futures ramifications (Planche 1, figs. 1, 2).

Culture de poumons entiers

Les organes sont prélevés à 5 jours d'incubation. Entre le deuxième et le quatrième jour de culture, nous constatons une croissance nette de l'épithélium. Le canal épithélial s'élargit beaucoup. Les dilatations deviennent de véritables sacs. De nouvelles digitations apparaissent: ce ne sont parfois que de simples plis de la paroi épithéliale mais bien plus souvent, elles constituent les ébauches des futures ramifications (Planche 1, fig. 3). Leur nombre, difficile à préciser (de 2 à 8), est fonction de la quantité de mésenchyme. L'histologie révèle une bonne différenciation de l'épithélium (Planche 1, fig. 4).

Une partie des cellules mésenchymateuses se concentre autour de l'épithélium au niveau des zones les plus actives. Le reste du mésenchyme se disperse plus ou moins.

Le développement d'un poumon embryonnaire explanté à 5 jours se poursuit

[1] Le liquide de Tyrode contient de la pénicilline G (sel de sodium) à raison de 10 unités par milieu de culture.

donc normalement en culture; il se poursuit d'autant mieux que le mésenchyme est plus abondant.

Culture de l'épithélium isolé

La séparation des ébauches épithéliales et mésenchymateuses est très délicate. Il peut arriver qu'un peu de mésenchyme reste adhérent à l'épithélium. Cependant, en aucun cas, nous n'avons observé de différenciation épithéliale. Il ne semble pas non plus y avoir de croissance. L'épithélium ne se ramifie jamais. Il conserve sa forme. Quelquefois il se tasse un peu en boule. Dans certains cas il s'étale. A l'histologie, on remarque assez souvent une différence entre les deux faces de l'épithélium: dans celle qui est tournée vers l'extérieur, les cellules sont très aplaties; elles sont au contraire plus saines, de type cylindrique ou cubique du côté du milieu et de la membrane vitelline où se trouve un peu de mésenchyme.

L'épithélium pulmonaire isolé en culture est donc incapable de se développer.

Réassociation de l'épithélium pulmonaire avec du mésenchyme homologue

Les organes, comme nous l'avons dit précédemment, son prélevés à 5 jours d'incubation. Nous utilisons le mésenchyme de quatre poumons de façon à former une masse suffisante autour de l'épithélium. L'explant subit une morphogénèse importante. Le canal épithélial s'élargit. L'épithélium pousse de nombreuses digitations (de 5 à 20) disposées de façon tout à fait anarchique (tableau 1). Ces digitations sont en plus grand nombre que celles formées pendant la culture d'un poumon entier. Ceci est dû vraisemblablement à la quantité de mésenchyme présent: celui-ci est plus abondant, comme nous l'avons indiqué, dans la cas d'une réassociation.

TABLEAU 1

Les résultats obtenus dans chaque type d'association

Type d'association		*Nombre total de cas**	*Nombre de survies*	*Différenciation positive de l'ép. pulm.*
Épithélium	*Mésenchyme*			
Pulmonaire	Pulmonaire	77	50	49+
Pulmonaire	Allantoïde	15	15	15−
Pulmonaire	Somites	56	56	56−
Pulmonaire	Méson. de 5 j.	23	22	22−
Pulmonaire	Méson. de 3 j.	6	6	6−
Pulmonaire	Métanéphros	17	17	15+

* Le plus grand nombre de nécroses dans les premières expériences est dû à ce que la technique n'était pas encore suffisamment au point.

L'épithélium est cylindrique avec des cellules hautes et serrées. Il s'est nettement différencié. Une partie du mésenchyme s'est concentrée autour de l'épithélium. Le reste est plus lâche ou dispersé (Planche 2, fig. 5).

La présence du mésenchyme pulmonaire apparaît donc indispensable à la croissance et à la différenciation de l'épithélium bronchique. Cependant il est possible que d'autres mésenchymes aient la même influence et puissent être substitués au mésenchyme pulmonaire.

Association de l'épithélium pulmonaire avec un mésenchyme hétérologue

L'épithélium pulmonaire a été associé à d'autres mésenchymes tels que ceux des somites (explantés à 3 jours), de la chorio-allantoïde (explantée à 5 jours) et du mésonéphros (explanté à 5 jours; le mésenchyme n'est pas pur, mais réparti entre les tubules épithéliaux; nous avons refait cette expérience en prélevant le mésenchyme à 3 jours) (tableau). L'épithélium n'a donné aucune morphogénèse (Planche 2, fig. 6). Il ne s'est pas ramifié. Ses cellules se sont aplaties. Le conduit épithélial allongé a gardé sa forme, quelquefois il s'est un peu gonflé. En certains cas, dans le mésenchyme somitique, il s'est transformé en vésicule (Planche 2, fig. 7). Enveloppé par la chorio-allantoïde, l'épithélium est resté sain et épais mais ne s'est pas ramifié.

Une association intéressante a été réalisée avec le mésenchyme métanéphrétique (explanté à 5 jours) (tableau). Celui-ci a une action morphogénétique nette; le canal épithélial se dilate et ébauche des digitations. Ces dernières sont moins nombreuses que celles qui sont produites sous l'influence du mésenchyme pulmonaire (leur nombre est difficile à préciser) (Planche 2, fig. 8). Leur aspect diffère aussi: elles sont plus arrondies et moins allongées; quelquefois, à un endroit quelconque du conduit épithélial, se forme une dilatation volumineuse à paroi très mince. Dans certaines portions, les cellules épithéliales sont aplaties et ne présentent aucun signe de différenciation. Dans d'autres régions, elles sont plus épaisses et légèrement différenciées. Le mésenchyme métanéphrétique se concentre en partie autour de l'épithélium, l'autre partie se dispersant.

Ces différents mésenchymes possèdent en commun la propriété d'empêcher la dispersion de l'épithélium pulmonaire. C'est ce que Grobstein (1953) a appelé 'l'effet antiétalement'. Ils n'ont aucune action organisatrice sauf le mésenchyme métanéphrétique. Ce dernier permet la ramification et, en partie, la différenciation de l'épithélium bronchique.

CONCLUSIONS

Comme Grobstein (1953), nous pouvons conclure que le mésenchyme est indispensable pour l'organogénèse de l'ébauche épithéliale. Grobstein a montré que l'épithélium de la glande salivaire de Souris a besoin d'un mésenchyme essentiellement spécifique. Dans nos expériences sur le poumon, la spécificité mésenchymale est un peu moins stricte, l'épithélium bronchique pouvant se ramifier et se différencier avec le mésenchyme métanéphrétique. Les autres mésenchymes se sont révélés incompétents.

On peut se demander si les mésenchymes pulmonaire et métanéphrétique agissent par l'intermédiaire d'une substance diffusant vers l'épithélium ou par un

effet de contact. C'est ce que nous tentons d'élucider en interposant une membrane entre ces deux constituants.

RÉSUMÉ

1. Nous recherchons comment se produit la différenciation du poumon embryonnaire de Poulet. Le poumon explanté à 5 jours d'incubation est dissocié en ses deux constituants, épithélium et mésenchyme, à l'aide de versène et de trypsine selon la méthode de Grobstein (1953). L'épithélium est mis en culture soit isolément soit réassocié à du mésenchyme homologue ou hétérologue.

2. Le poumon non dissocié de Poulet continue son développement en culture et ceci d'autant mieux que la quantité de mésenchyme présent est plus grande.

3. L'épithélium isolé est incapable de poursuivre sa morphogénèse.

4. L'épithélium réassocié à du mésenchyme pulmonaire se différencie et se ramifie de façon importante.

5. Des mésenchymes hétérologues comme ceux du mésonéphros, des somites et de la chorio-allantoïde empêchent la dispersion de l'épithélium. Ils permettent sa survie mais ils n'ont aucune action morphogénétique. Le mésenchyme métanéphrétique au contraire favorise la ramification de l'ébauche épithéliale et partiellement sa différenciation.

SUMMARY

1. The work reported here is concerned with the differentiation of the embryonic lung of the chick. The lung at 5 days of incubation was cultured; or it was dissociated with versene and trypsin by Grobstein's method and the epithelium then cultured either alone or associated with homologous or heterologous mesenchyme.

2. The undissociated lung continued its development in culture; the more mesenchyme was present the better was its development.

3. The isolated epithelium was unable to continue its morphogenesis.

4. The epithelium reassociated with lung mesenchyme differentiated and ramified significantly.

5. Heterologous mesenchyme from mesonephros, somites, or chorio-allantois prevents spreading of the epithelium and permits its survival, but has no morphogenetic action. Metanephric mesenchyme on the contrary favours the branching of the epithelial rudiment, and to some extent its differentiation.

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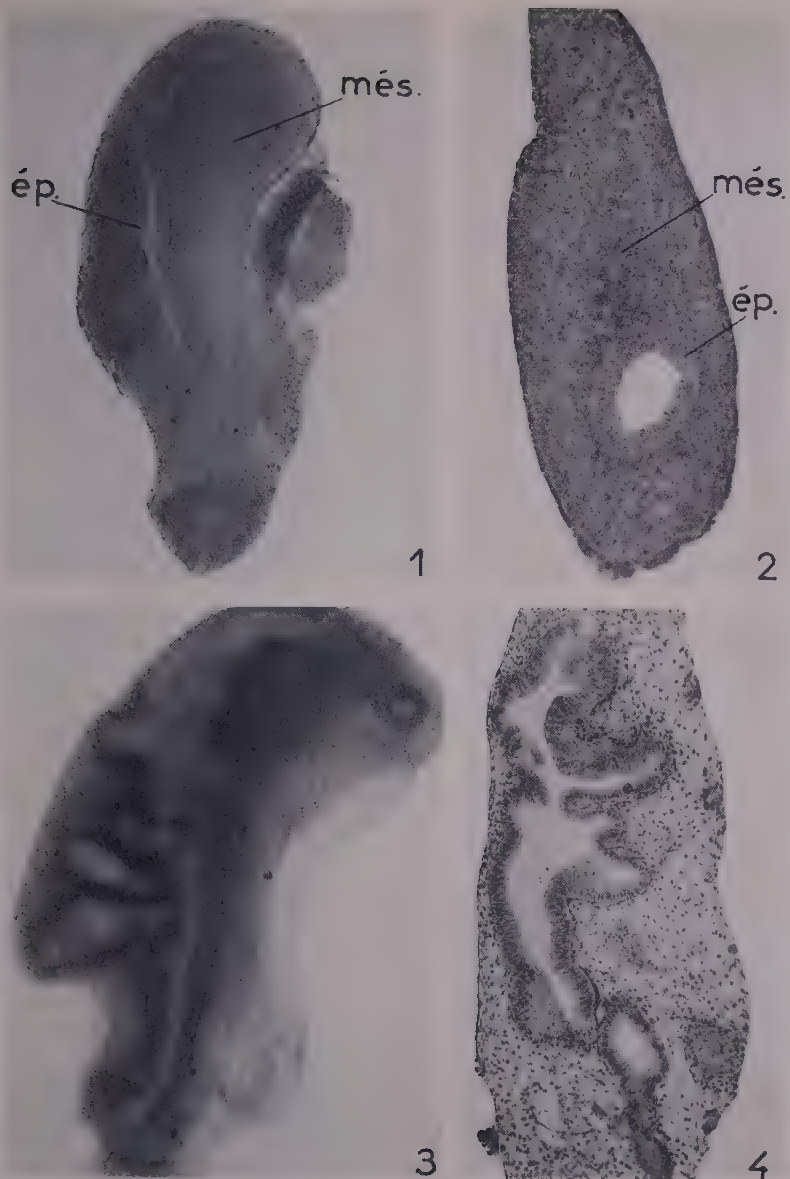

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Planche 1

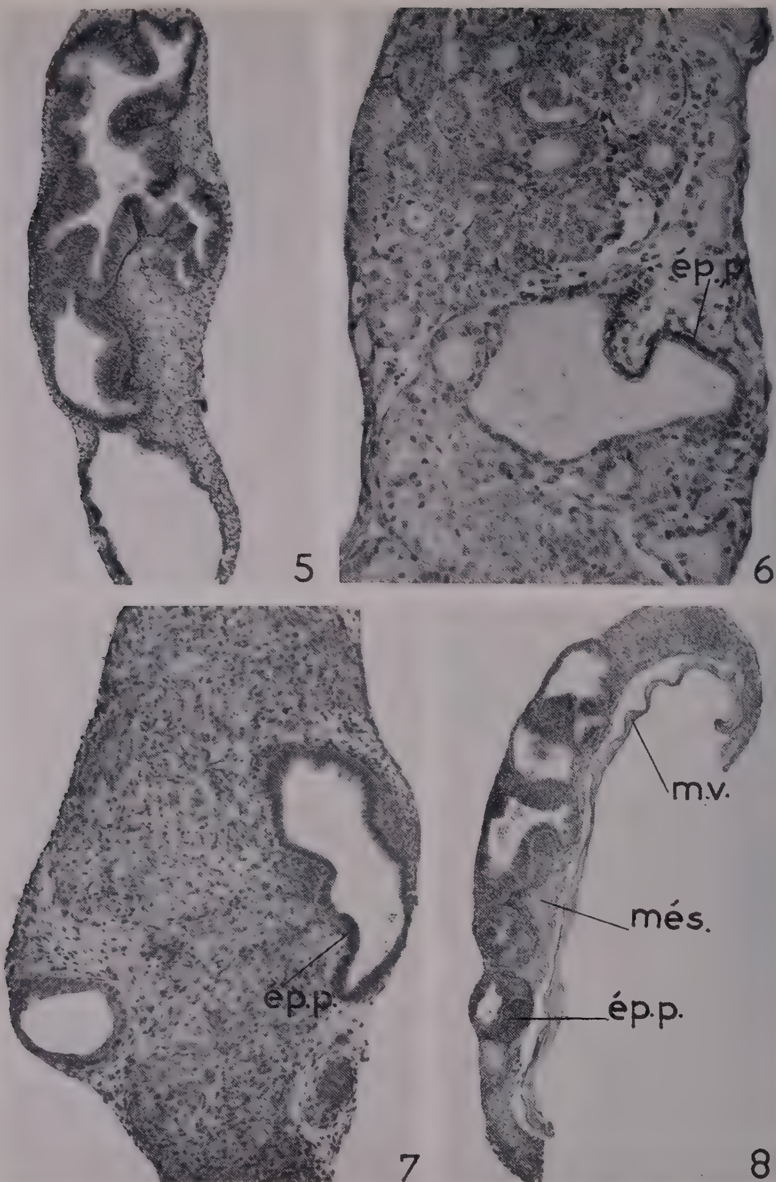

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Planche 2

EXPLICATION DES PLANCHES

PLANCHE 1

FIG. 1. Poumon explanté à 5 jours et fixé immédiatement. Préparation au Carmin chlorhydrique alcoolique. *ép.*, épithélium; *més.* mésenchyme. ×94.

FIG. 2. Coupe d'un poumon explanté à 5 jours et fixé immédiatement. *ép.*, épithélium; *més.*, mésenchyme. ×240.

FIG. 3. Poumon explanté à 5 jours et cultivé pendant 5 jours. Préparation au Carmin chlorhydrique alcoolique. ×94.

FIG. 4. Coupe d'un poumon explanté à 5 jours et cultivé pendant 5 jours. ×200.

PLANCHE 2

FIG. 5. Coupe d'une réassociation d'épithélium et de mésenchyme pulmonaires (explantés à 5 jours) cultivée pendant 5 jours. ×150.

FIG. 6. Coupe d'une association d'épithélium pulmonaire et de mésonéphros (explantés à 5 jours) cultivée pendant 5 jours. *ép.p.*, épithélium pulmonaire. ×375.

FIG. 7. Coupe d'une association d'épithélium pulmonaire (explanté à 5 jours) et de mésenchyme somitique (explanté à 3 jours) cultivée pendant 5 jours. *ép.p.*, épithélium pulmonaire. ×240.

FIG. 8. Coupe d'une association d'épithélium pulmonaire et de mésenchyme métanéphrétique (explantés à 5 jours) cultivée pendant 5 jours. *ép.p.*, épithélium pulmonaire; *més.*, mésenchyme; *m.v.*, membrane vitelline. ×138.

(*Manuscript received 27: iii: 61*)

Transfer of Primordial Germ-cells in *Xenopus laevis*

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WITH TWO PLATES

INTRODUCTION

THERE have been many claims for the segregation of Anuran primordial germ-cells at an early embryonic stage. Most authors agree that these cells may be distinguished with ease in the most dorsal region of the larval endoderm and, somewhat later in development, at the base of the dorsal mesentery and in the undifferentiated gonad (see review by Johnston, 1951). Bounoure (1934) and Blackler (1958) claim to have traced the origin of the primordial germ-cells as early in development as the late blastula stage and to have recognized cell inclusions that become restricted to the germ line at all stages between the fertilized egg and the late blastula.

As pointed out by Everett (1945), some workers in this field of embryological study have firmly denied the existence of primordial germ-cells, while others have been cautious of accepting the principle that these cells give rise to any of the definitive sex-cells (gametes). The experimental studies reported in this paper present a proof that not only do primordial germ-cells actually exist in late neurula stages but that at least some of these cells are directly ancestral to some of the functional gametes.

The history of the primordial germ-cells in the South African Clawed Toad, *Xenopus laevis*, has already been documented by Nieuwkoop (1956 *a*, *b*) and Blackler (1958) and, in view of such other considerations as ease of rearing and breeding and the research programme of this laboratory, it was decided to employ embryonic material of this species.

The technique of transfer of the primordial germ-cells, described below, is a technique whereby germ-cells of embryo A can develop within embryo B. The application of the technique, as reported here, is concerned with aspects of the germ-cell problem, but it may be applied also to problems such as sex-reversal and the production and genetic analysis of abnormal embryos after nuclear transplantation (see Fischberg, 1960).

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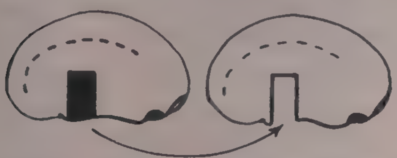
MATERIALS AND METHODS

In the light of our knowledge of *Xenopus* germ-cells there appear to be three stages in development which offer the most likely chances of transfer success, as outlined by Blackler (1960). All transfers commented on in this present paper were effected using neurulae of stages 19–26 (Nieuwkoop & Faber, 1956).

The primordial germ-cells of the stage 23 neurula can be readily seen in sections stained by the Altmann–Volkonsky method. Their cytoplasm possesses the characteristic 'cytoplasme germinal' of Bounoure (1934), which is distributed adjacent to the nucleus and also apposed to the cell membrane. As may be seen in Plate 1, fig. A, the cells are quite closely aggregated—a point at variance with the situation in *Rana temporaria*—and situated deep in the posterior endoderm about 0·6 mm. anterior to the hind-end of the embryo (i.e. just anterior to the anus). They number between 20 and 30 cells.



TEXT-FIG. 1. Scheme of germ-cell transfer operation.

After the decapsulation of both host and donor neurulae in full-strength Niu and Twitty solution, the endodermal region containing the primordial germ-cells of the host (together with the overlying ventral mesoderm and ectoderm) is excised and replaced by the same region taken from the donor embryo. Text-fig. 1 indicates the scheme of the operation and the size of the piece removed or grafted.

The grafted material is kept in position by a glass bridge and may take 10 minutes or more to heal in place sufficiently to allow removal of the bridge. When the edges of the graft are almost obliterated by ectodermal overgrowth, the host is transferred to 0·1 Niu and Twitty solution. Here it must be stressed that ectodermal overgrowth is not to be taken as an indication that the graft has been harmoniously incorporated. Graft breakdown may occur as late in development as the stage 40 tadpole. The appearance of the host embryo after healing is shown in Plate 2, fig. D.

Only 40 per cent. of all transfers made were successful in that not only did the graft heal in place but the host continued to develop and began feeding normally. In later experimental series, not reported here, we have obtained 70–90 per cent. success in transfers, demonstrating that the skill of the operator is also involved as an important factor for success.

It will be appreciated that the removal of the host germ-cell region should theoretically result in complete host sterility. However, it is more reasonable to suppose that in practice some of the host's germ-cells may not be removed during the operation: it is thus necessary to ensure that grafted germ-cells can be distinguished from any remaining host germ-cells. In this respect, the use

of the nuclear marker discovered by Fischberg (see Elsdale, Fischberg, & Smith, 1958) has allowed such a distinction to be made.

In a normal embryo of *X. laevis* all of the constituent cells potentially bear two nucleoli per nucleus, although some may contain only one nucleolus, probably the result of fusion. Such embryos can be termed $2N$. In embryos *heterozygous* for the nuclear marker, *every* cell has a nucleus with only one nucleolus. These marked embryos can be termed $1N$.

Plate 1, figs. B, C, show cells of a normal ($2N$) and a marked ($1N$) tadpole. The mutation behaves in a simple Mendelian manner so that, when a $1N$ frog (of either sex) is mated with a $2N$ frog, half the offspring are 1-nucleolate and half are 2-nucleolate (actually, 48·5 per cent. $1N$: 51·5 per cent. $2N$ in a multi-sample analysis of over 550 offspring (Fischberg & Wallace, 1960)).

When germ-cells from a normal ($2N$) neurula are grafted into a marked ($1N$) neurula and the host gonads later examined microscopically, any $2N$ cell observed must be necessarily of graft origin. It is, however, not possible to determine whether cells carrying a single nucleolus are of host or graft origin.

$$\begin{array}{ccccc}
1, 1 & & 2N & & 1, 1 \\
\text{gametes} & \longrightarrow & \text{zygotes} & \longleftarrow & \text{gametes} \\
\uparrow & & & & \uparrow \\
\text{meiosis} & & & & \text{meiosis} \\
\uparrow & & & & \uparrow \\
2N\ g\text{–}c \text{ in } 1N \text{ host} & \ldots & \times & \ldots & 2N \text{ normal} \\
\downarrow & & & & \downarrow \\
\text{meiosis} & & & & \text{meiosis} \\
\downarrow & & & & \downarrow \\
\text{gametes} & & \text{zygotes} & & \text{gametes} \\
1, 0 & \longrightarrow & 2N, 1N & \longleftarrow & 1, 1
\end{array}$$

(*a*)

$$\begin{array}{ccccc}
1, 0 & & 2N, 1N & & 1, 1 \\
\text{gametes} & \longrightarrow & \text{zygotes} & \longleftarrow & \text{gametes} \\
\uparrow & & & & \uparrow \\
\text{meiosis} & & & & \text{meiosis} \\
\uparrow & & & & \uparrow \\
1N\ g\text{–}c \text{ in } 2N \text{ host} & \ldots & \times & \ldots & 2N \text{ normal} \\
\downarrow & & & & \downarrow \\
\text{meiosis} & & & & \text{meiosis} \\
\downarrow & & & & \downarrow \\
\text{gametes} & & \text{zygotes} & & \text{gametes} \\
1, 1 & \longrightarrow & 2N & \longleftarrow & 1, 1
\end{array}$$

(*b*)

TEXT-FIG. 2. (*a*) Shows the expected results when a $2N$ germ-cell graft in $1N$ host (whose own germ-cells have not been entirely removed) is mated with a normal frog. No offspring can be directly ascribed to the graft cells, but their presence is manifest by the excess of $2N$ offspring over $1N$ offspring. (*b*) Shows the expected results when a $1N$ germ-cell graft in $2N$ host (whose own germ-cells have not been entirely removed) is mated with a normal frog. All $1N$ offspring are of graft origin, while an equal number of the $2N$ offspring are also of graft origin.

For the rest, the technique of germ-cell transfer must ensure that the grafted cells are capable of developing into functional gametes. Some experimental animals were raised to sexual maturity, and in these both possible combinations of the nuclear marker have been employed. The 1*N*-in-2*N* combination is superior since it is only by the mating of adult frogs of this character that certain offspring may be ascribed unreservedly to the grafted material. Text-fig. 2 shows how the percentage of graft success may be calculated from inspection of the offspring derived from the mating of normal (2*N*) frogs with experimental frogs of either combination.

RESULTS OF GERM-CELL TRANSFERS

Histological

Three marked (1*N*) animals into which an unmarked (2*N*) graft had been made at the neurula stage were killed at completion of their metamorphosis and their kidneys and attached gonads fixed and sectioned. All 3 pairs of gonads possessed some gonocytes of 2*N* type (see Plate 2, figs. E–H). In the females, many oocytes were beginning their growth phase, making the nucleolar count impossible, but it was easy to assess the number of nucleoli in the less-developed oogonia.

Breeding

Twelve experimental frogs were raised to sexual maturity and mated to unmarked normal frogs according to the scheme of Text-fig. 2.

TABLE 1

Results of mating 1*N frogs, possibly containing* 2*N graft germ-cells, with* 2*N frogs. Percentage graft success is calculated from excess of* 2*N over* 1*N offspring*

Frog	*Sex*	*No. of offspring analysed* (*x*)	*No. of* 1*N offspring* (*y*)	*No. of* 2*N offspring* (*z*)	% *graft success** $\frac{(z-y)100}{x}$
1	♀	108	53	55	1·8
2	♂	200	89	111	11·0
3	♂	200	2	198	98·0

* The graft success is calculated on the assumption that the offspring of a mating 1*N*×2*N* are in the ratio of 50 1*N* : 50 2*N* per 100 embryos. Actually, there are always slightly more 2*N* offspring than 1*N* (see p. 636); thus the figures quoted for graft success are slightly in excess of the correct percentage (e.g. for frog 1, the graft success is zero).

Three of these frogs were of the 2*N*-in-1*N* combination and the results of their matings with normal (2*N*) frogs are summarized in Table 1.

The remaining 9 frogs were of the 1*N*-in-2*N* combination and have been mated with 2*N* frogs. All the 1*N* offspring can be ascribed directly to the grafted

material, and an equivalent number of the $2N$ offspring are of like source although they cannot be distinguished from those $2N$ offspring derived from host germ-cells remaining after the operation. The results of these matings are summarized in Table 2.

TABLE 2

Results of mating 2N frogs, possibly containing 1N graft germ-cells, with 2N frogs. Percentage graft success is calculated from the number of 1N offspring

Frog	Sex	No. of offspring analysed (x)	No. of $1N$ offspring (y)	No. of $2N$ offspring (z)	% graft success* $\frac{(2y)100}{x}$
4	♀	100	0	100	0·0
5	♂	200	92	108	92·0
6	♂	200	89	111	89·0
7	♂	75	0	75	0·0
8	♂	200	95	105	95·0
9	♀	125	0	125	0·0
10	♂	171	72	99	84·2
11	♂	200	96	104	96·0
12	♀	75	0	75	0·0

* The graft success is calculated on the assumption that the offspring of a mating $1N \times 2N$ are in the ratio of 50 $1N$: 50 $2N$ per 100 embryos. Actually, there are always slightly fewer $1N$ offspring than $2N$ (see p. 636); thus the figures quoted for graft success are slightly lower than the correct percentage (e.g. for frogs 8 and 11, the graft success is 100 per cent.).

DISCUSSION

The experiments described above demonstrate that grafts of posterior endoderm containing the so-called primordial germ-cells can be successfully carried out between embryos of *Xenopus* at the neurula stage. The healing process is facilitated if the ecto-mesodermal coat of the ventral endoderm is transferred as well, but its presence is not essential for success. It is clear that some cells of the grafted material, in successful operations, make their way into the host gonadal ridges and here are able to perform the differentiation necessary for the production of functional eggs and sperm. However, it must be readily admitted that it is not possible to state if the graft gametes are derived from some of the transplanted endodermal cells, from the so-called primordial germ-cells described by Bounoure and Blackler, or from both sources. Nevertheless, there are reasons, which derive their force from being taken together, for supposing that only the primordial germ-cells are involved. In the first place, the technique was specifically designed to ensure, as far as possible, that cells containing the 'cytoplasme germinal' were transferred. Secondly, if the grafted endoderm (whose cells are much more numerous than the primordial germ-cells) provided the functional sex-cells, it is difficult to interpret those experimental cases in which the graft healed in perfectly but none of its cells gave rise to gametes.

Moreover, there is no reason to suppose that if 'germinal' endoderm cells exist they have the same distribution and aggregation as the primordial germ-cells.

In the experiments, care was taken at the time of operation to graft pieces of endoderm very similar in size. In view of the argument at the end of the last paragraph, one is naturally inclined to ask why the graft is successful in some cases and not in others. We would draw attention to the significant result that graft success is either very high or, with the exception of one case, very low indeed. This result is what one might expect if the primordial germ-cells are so closely aggregated that small differences in the size of the piece grafted would determine whether or not they were included. A comparison of Text-fig. 1 and Plate 1, fig. A shows that the primordial germ-cells lie very near the dorsal periphery of the grafted region. It is impossible, of course, to cut pieces of *exactly* the same size, and thus justifiable to expect the clump of germ-cells to be wholly included in the graft or to be wholly excluded from it.

Moreover, we feel that the embryonic stage of the donor must be considered. As shown in Plate 1, fig. A, the primordial germ-cells are already quite dorsal in the posterior endoderm of neurulae at stage 23. Some of the donor neurulae used in the experiments were already at stage 26, and it is quite possible that when grafting from such late neurulae the primordial germ-cells have moved too far dorsally to be included in the piece excised. Unfortunately, the recipient embryos were not maintained in isolation, through considerations of space, from the time of operation to sexual maturity, and the records have become, in consequence, somewhat less detailed. An inspection of the protocols suggests a rough concordance between the number of neurulae, later than stage 23, used as donors and the number of frogs showing no graft success, but this should be regarded only as an indication, and no proof, of the interpretation advanced earlier in this paragraph. Anyone who cares to repeat, or utilize, the technique for neurula stages is advised not to use as donor material any embryo later than stage 23 of the normal table.

Whatever view one likes to take in this matter, it is certain that at least some of the definitive gametes are the descendants of cells located in the posterior endoderm of neurulae. While the 'cytoplasme germinal' is a very useful cellular marker for recognizing the primordial germ-cells in sections of fixed embryonic material, the experiments reported here do not in any way support the idea that the 'cytoplasme germinal' acts as a germ-cell determinant. This is a different problem, requiring an extensive experimental analysis not so far performed.

No graft success has been obtained with mature female animals in these studies (see Tables 1 and 2), although graft oocytes have been detected in the sectioned material. This failure is of no significance, since in further work, using the technique but for a differently designed experiment, we found that females were quite capable of laying fertilizable eggs derived from grafted primordial germ-cells.

It is noticeable that in those experimental cases where graft success has been nil, the frogs, nevertheless, produce numerous gametes of host origin. Sterilization of the host during the operation is thus not always achieved, and the gametes produced subsequently may have been derived from an unreduced number of host primordial germ-cells or from a reduced number of these cells that have subsequently regulated by an increase in their mitotic rate. This latter situation may be compared with that described by Bounoure, Aubry, & Huck (1954) in which the primordial germ-cells, reduced in number by ultra-violet irradiation, nevertheless, completely repopulate the gonad. We feel the character of our experiments was not such as to justify raising again the issue of metaplasia to explain the failures.

In conclusion we believe that (1) primordial germ-cells definitely exist in neurula stages of *X. laevis*, and (2) these cells are directly ancestral to at least some of the definitive gametes. The possibility remains that some of the gametes are derived from some secondary source, though this seems unlikely in view of the high percentage of graft success in successful 'takes'.

SUMMARY

1. A technique is described whereby the so-called primordial germ-cells of *X. laevis*, together with some accompanying non-germinal cells, may be grafted from one embryo to another at the neurula stage. A nuclear marker enabled graft cells to be identified.

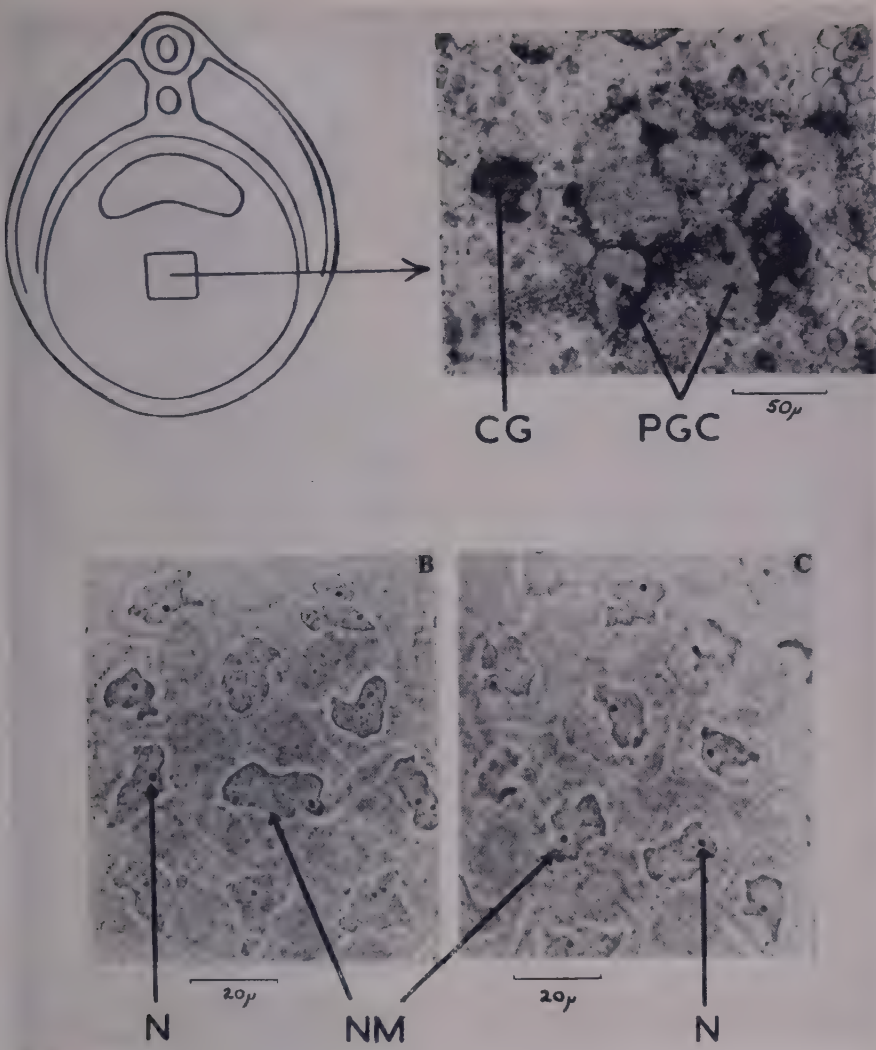
2. Some cells from the graft migrate into the host gonads and there form functional gametes. It is concluded that primordial germ-cells actually exist in neurula stages, that these cells are probably identical with the so-called primordial germ-cells described in histological studies, and that they are directly ancestral to at least some of the definitive eggs and sperm.

RÉSUMÉ

Migration de cellules germinales primordiales chez Xenopus laevis

1. Une technique est décrite dans laquelle les prétendues cellules germinales primordiales de *Xenopus laevis*, ainsi que quelques cellules somatiques qui les accompagnent, peuvent être greffées d'un embryon à l'autre au stade neurula. Un marqueur nucléaire permet de reconnaître les cellules greffées.

2. Quelques cellules du greffon migrent dans les gonades de l'hôte et y donnent naissance à des gamètes fonctionnels. La conclusion indique que les cellules germinales primordiales existent au stade neurula, que ces cellules sont probablement identiques aux prétendues cellules germinales primordiales décrites dans les études histologiques, et qu'elles sont directement à l'origine d'une partie au moins des ovules et des spermatozoïdes définitifs.



A. W. BLACKLER *and* M. FISCHBERG

Plate 1

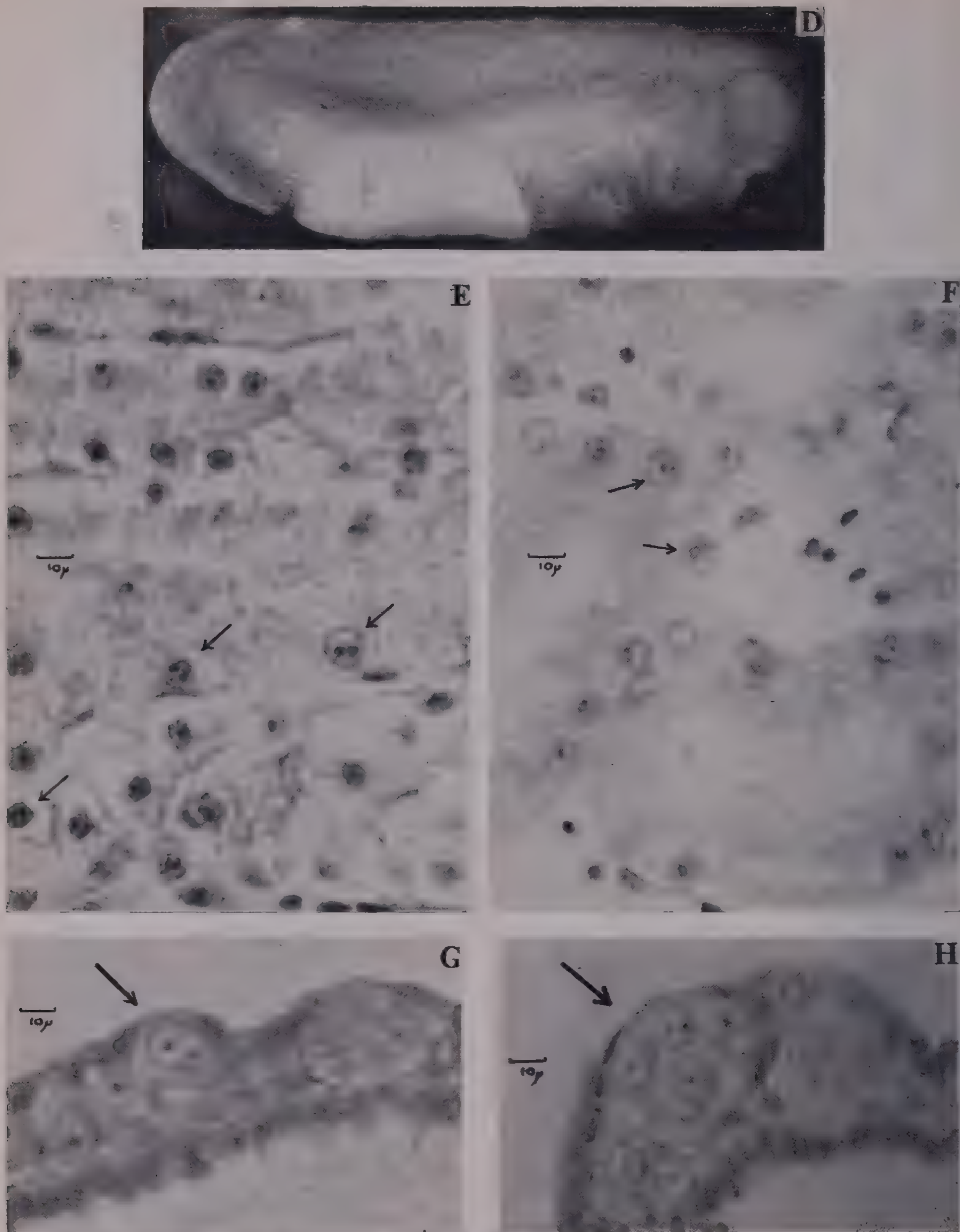


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Plate 2

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EXPLANATION OF PLATES

PLATE 1

FIG. A. Part of a transverse section through a stage 23 neurula to show the closely aggregated primordial germ-cells of *Xenopus*. CG, 'cytoplasme germinal'; PGC, primordial germ-cells.

FIGS. B, C. Phase-contrast preparations of epidermal cells of the tadpole tail in a normal (*B*) and a marked (*C*) larva. The nucleoli have been stained with pyronin for greater clarity. N, nucleoli; NM, nuclear membranes.

PLATE 2

FIG. D. Photograph of experimental animal 4 hours after operation. Considerable elongation has taken place in both graft and host.

FIG. E. Section through kidney of unmarked control toad to show the typically 2-nucleolate condition.

FIG. F. Section through kidney of a female experimental $2N$-in-$1N$ toad to show the 1-nucleolate condition of the host.

FIG. G. Section through the host gonad of the toad, whose kidney is shown in fig. F, to show the presence of a grafted 2-nucleolate gonocyte (arrow).

FIG. H. Section through the same gonad as fig. G to show a nest of grafted 2-nucleolate oogonia (arrow).

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Transplantation et Régénération chez la Planaire *Dendrocoelum lacteum*

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AVEC UNE PLANCHE

INTRODUCTION

PLUSIEURS articles ont été consacrés ces deux dernières années au pouvoir régénérateur restreint de la grande planaire non pigmentée *Dendrocoelum lacteum* Oerst. (*Planaria lactea* O.F.M.) et de son équivalent américain *Procotyla fluviatilis* (Leidy), qui fut confondue avec la précédente dans les travaux antérieurs à 1930. La tête ne se régénère pas chez ces formes à partir d'une section qui passe en arrière de la racine pharyngienne, alors que la queue est reconstituée à partir de n'importe quel niveau (Lillie, 1901). Dans la région prépharyngienne elle-même, la qualité et la vitesse de la régénération décroissent de l'avant vers l'arrière, suivant un gradient dont Sivickis (1931) a établi la courbe. Curtis & Schulze (1924) ayant fait un dénombrement approximatif des cellules de régénération (qu'ils appellent 'formative cells') chez *Planaria maculata* (*Dugesia tigrina*), planaire à régénération totale, et *Procotyla fluviatilis*, constatent que le parenchyme de la première espèce est environ trois fois plus riche en ces cellules que celui de la seconde espèce. Ils suggèrent que le pouvoir de régénération pourrait être directement fonction de la richesse du parenchyme en cellules de régénération et qu'en dessous d'un certain seuil la régénération serait impossible.

Une étude quantitative précise montre que la densité moyenne du parenchyme en néoblastes (c'est-à-dire en cellules de régénération) est effectivement moins forte chez *Dendrocoelum lacteum* (Stéphan-Dubois, 1961) que chez *Dugesia lugubris* (Lender & Gabriel, 1960) dont le pouvoir de régénération est total. Mais cette différence n'est pas grande, puisqu'elle est de l'ordre de 7 néoblastes chez la première espèce pour 10 chez la seconde. De plus la numération des néoblastes à différents niveaux antéro-postérieurs du corps indique que la variation du nombre des néoblastes (nombre absolu ou densité par unité

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de volume) ne justifie pas la perte brutale du pouvoir de régénération céphalique dès l'apparition du pharynx.

Ces observations histologiques concordent avec les recherches expérimentales qui ont pour base l'action des rayons X sur les néoblastes (Kolmayer & Stéphan-Dubois, 1960): en combinant l'irradiation de la région prépharyngienne avec une amputation au niveau des yeux, on démontre que les cellules de régénération de la région moyenne ou postérieure sont capables de traverser la région irradiée et de régénérer une tête normale.

Dans la présente note, nous apportons quelques compléments aux résultats précédents, à la suite d'expériences nouvelles qui combinent les techniques déjà utilisées avec des transplantations.

TECHNIQUES

Les techniques d'amputations, d'anesthésie et d'irradiation aux rayons X ne diffèrent pas de celles qui ont été publiées en 1960. Nous avons remarqué depuis lors que les *Dendrocoelum lacteum*, vite lésées par les anesthésiques, même par le chlorétone (acétone chloroforme), supportent mieux celui-ci quand sa solution est vieille de quelques mois.

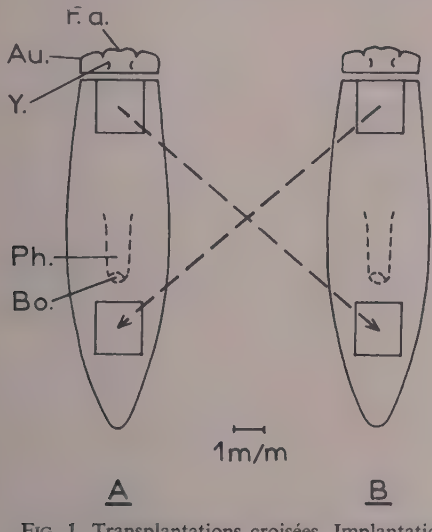


FIG. 1. Transplantations croisées. Implantation d'un greffon d'origine prépharyngienne dans la région postérieure. *Au.*, auricules; *Bo.*, bouche; *F.a.*, fossette adhésive; *Ph.*, pharynx; *Y.*, yeux.

Des deux techniques de transplantation utilisées: celle des autogreffes avec coloration vitale (du greffon ou de l'hôte) ou celle des homogreffes sans coloration vitale, la première n'a servi qu'à produire quelques témoins car la mortalité est trop considérable, même si le colorant est très dilué.

Les sujets destinés aux homogreffes sont dans certains cas nourris rien qu'avec

des Tubifex ou rien qu'avec des Aselles, de sorte que leur contenu intestinal se colore de façon durable en rougeâtre ou en gris, ce qui permet de pallier l'absence de pigments naturels ou de colorant vital et de suivre plus facilement le devenir des greffons. Les transplantations sont parfois croisées (fig. 1) pour économiser un matériel assez rare; les sujets sont décapités avant la greffe pour éviter autant que possible les contractions qui nuiraient à la prise du greffon. Quelques hôtes non décapités et ne servant pas de donneur témoignent que les résultats des expériences sont identiques dans les deux cas. Seuls sont conservées les pièces dans lesquelles le greffon s'est bien soudé à l'hôte par ses 4 faces, donc sans laisser de bords libres.

Transplantation d'un greffon d'origine prépharyngienne dans la région postérieure

Cette expérience (fig. 1) fut primitivement tentée dans le but de provoquer une régénération céphalique en arrière du pharynx, en activant les tissus postérieurs par un territoire antérieur. Si les résultats envisagés sous cet angle sont négatifs, ils sont intéressants à d'autres points de vue.

1ère série: la section passe par le greffon

Après la transplantation, une section transversale supprime la partie antérieure du greffon.

Dans un premier groupe, les 12 fragments postérieurs évoluent tous de la façon suivante (fig. 2): la partie libre du greffon se cicatrise, s'allonge légèrement

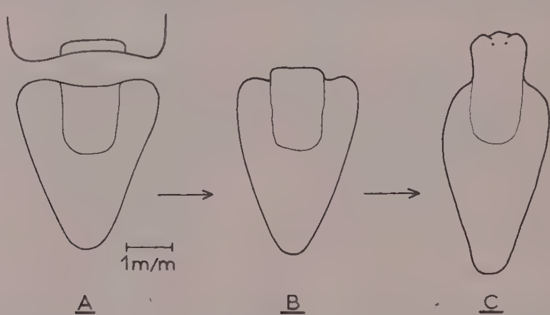


FIG. 2. Greffon d'origine prépharyngienne implanté dans la région caudale. *A*, une section transversale supprime la partie antérieure du greffon. *B*, Le greffon s'allonge légèrement. Les bords de l'hôte s'arrondissent. *C*, Le greffon régénère une tête complète. Les bords de l'hôte ne participent pas à la régénération (9e jour après la section).

et régénère par épimorphose une petite tête typique. La régénération est rapide, comme dans tous les cas où la section passe juste en arrière des yeux anciens. Les bords blessés de l'hôte se cicatrisent eux aussi, mais ils se fondent avec le greffon sans participer à la régénération. L'ensemble reprend la forme d'une petite planaire dominée par la partie céphalique du greffon.

Dans un second groupe de 15 sujets, les donneurs sont entièrement irradiés aux rayons X. Leurs néoblastes sont donc incapables de se différencier en un régénérat et les greffons issus de ces donneurs ne sont pas aptes à se régénérer par eux-mêmes. Effectivement il apparaît d'abord, en avant des greffons cicatrisés, le mince liseré d'un blastème abortif et vite nécrosé. Dans 6 cas ce liseré est peu à peu coiffé par les bords cicatriciels de l'hôte et le fragment reste dans cet état jusqu'à sa mort (fig. 3A, B). Dans les 9 autres cas (fig. 3A, C), les bords de l'hôte restent en place. Le bord cicatrisé du greffon est libre; vers la fin de la 2ème semaine il se garnit d'un liseré qui, cette fois, se transforme en

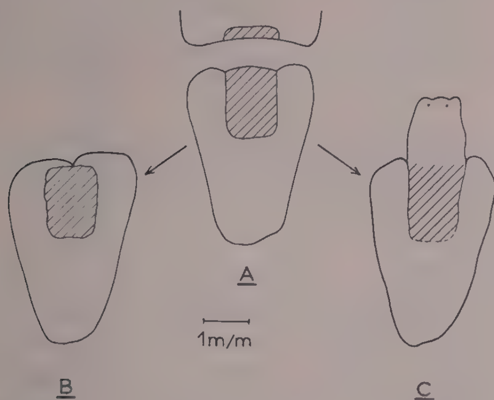


FIG. 3. Greffon irradié d'origine prépharyngienne, implanté dans la région caudale. *A*, une section transversale supprime la partie antérieure du greffon. *B*, cas où les bords de l'hôte encerclent le greffon. Pas de régénération possible. *C*, cas où le bord antérieur du greffon reste libre. Les néoblastes irradiés du greffon sont remplacés par les néoblastes sains de l'hôte. La régénération céphalique du greffon est possible quand ces néoblastes sains arrivent contre le bord blessé (23e jour après la section).

un blastème sain puis en une petite tête normale. Si le délai de régénération est si long, c'est qu'il a fallu aux néoblastes sains de la région postérieure une douzaine de jours pour traverser le greffon irradié. Malgré ce délai, les bords de la plaie qui appartiennent au territoire postérieur n'ont pas régénéré. Les cellules de régénération nécrosées du greffon sont, elles aussi, remplacées et le greffon redevenu sain et oculé prend la dominance du fragment qui évolue vers une planaire normale (Planche, fig. A).

Ces résultats confirment et complètent ceux que les expériences d'irradiations régionales avaient mis en évidence, à savoir la totipotence et le pouvoir migrateur des cellules de régénération de la région postérieure à la racine pharyngienne. Dans le cas des transplantations, il s'agit plus particulièrement des cellules de la région des organes copulateurs. A aucun niveau les *D. lacteum* ne sont donc dépourvues de néoblastes totipotents.

2ème série: la section passe en avant du greffon

Après la transplantation, la section transversale passe entre le pharynx et le bord antérieur du greffon (fig. 4A; 15 cas). Ce bord n'est donc plus libre comme dans le cas précédent et le greffon ne peut régénérer tout en prenant la dominance. On pourrait donc espérer une induction provoquée par le greffon et favorable à la régénération céphalique du territoire postérieur blessé. Il n'en est rien: l'hôte se cicatrise mais ne régénère pas. La face dorsale du greffon se bombe tandis que la face ventrale s'invagine. Des yeux apparaissent sur la proéminence dorsale 15 à 30 jours après l'opération (fig. 4B, C). Les fragments ne redeviennent pas des planaires normales mais sont néanmoins polarisés par le greffon dont les mouvements conditionnent ceux de la petite planaire toute entière.

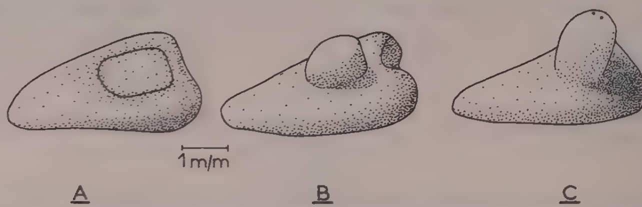


Fig. 4. Greffon d'origine prépharyngienne, implanté dans la région caudale. *A*, la section passe en avant du greffon. *B*, le greffon se bombe dorsalement. *C*, le greffon possède deux yeux (25e jour après la section).

Transplantation d'un greffon d'origine postpharyngienne dans la région antérieure d'un hôte irradié

L'hôte est décapité puis irradié totalement aux rayons X. Un rectangle de tissu d'origine postpharyngienne, greffé dans la région antérieure, représente le seul stock de cellules de régénération capables d'édifier un régénérat. Comme aucune section dans la région postérieure ne provoque la migration de ces néoblastes vers la queue, celle-ci se nécrose peu à peu; mais seule l'évolution de la région prépharyngienne nous intéresse dans cette série.

Dans 5 cas qui servent de témoins, le greffon est laissé intact (fig. 5A). Un petit blastème vite nécrosé se forme en avant, à partir des cellules de régénération irradiées. Ce blastème disparaît et chez les 3 sujets survivants se reforme vers le 20ème jour, à partir des néoblastes sains du greffon, un blastème sain qui se différencie en une tête complète.

Dans 16 autres cas, la transplantation est suivie le lendemain d'une section transversale qui passe environ au quart antérieur du greffon (fig. 5B). Chaque planaire est ainsi coupée en deux fragments de taille très inégale.

Les petits fragments antérieurs sont fragiles et 12 d'entre eux périssent en trois jours. Les 4 survivants régénèrent sans retard une queue vers l'arrière,

à partir du greffon. La cicatrice antérieure se garnit pendant quelques jours d'un liseré de cellules radiolésées; elles disparaissent pour faire place à un blastème sain qui se différencie vers le 18ème jour en une tête complète. Ces 4 cas méritent une attention spéciale car ils prouvent qu'un très petit fragment de territoire postérieur suffit à reconstituer un blastème caudal, à fournir assez de cellules pour un blastème céphalique et à réparer les tissus radiolésés qui l'environnent (Planche, fig. B). On peut en être surpris à première vue. En réalité, en se basant sur le dénombrement des cellules de régénération effectué sur cette espèce, on peut évaluer leur nombre à quelque 40.000 dans le fragment de greffon.

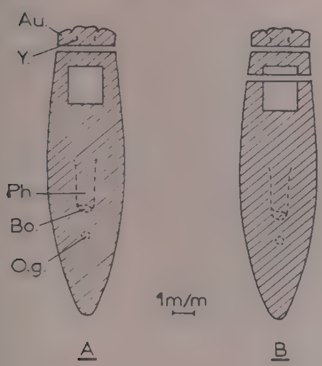


FIG. 5. Greffon sain d'origine postpharyngienne implanté derriere les yeux d'un hôte irradié aux rayons X et décapité avant la transplantation. *A*, la transplantation n'est pas suivie d'une 2e amputation. *B*, la transplantation est suivie d'une 2e amputation qui partage l'hôte et le greffon en un court fragment antérieur et un long fragment postérieur. Même légende qu'en fig. 1; et *O.g.*, orifice génital.

Parmi les 16 grands fragments postérieurs de cette expérience, 5 ne peuvent évoluer parce que la plaie se rétracte en coin puis se soude à elle-même, ce qui exclut toute possibilité de régénération. Les bords blessés des 11 autres restent libres; le greffon ne régénère rien. Les traces de radio-lésions visibles sur les parties latérales de l'hôte disparaissent en deux semaines. Dans un seul cas l'hôte régénère une tête latérale typique (Planche, fig. C).

CONCLUSIONS

Les résultats qui viennent d'être décrits confirment les conclusions tirées d'un précédent travail: à savoir que la région postérieure de *D. lacteum*, région normalement incapable de régénérer une tête, contient cependant des néoblastes susceptibles de migrer vers l'avant. Ceux-ci vont coloniser une région prépharyngienne radio-lésée, lui permettant ainsi d'exprimer ses potentialités régénératrices, supprimées par l'irradiation. Ces conclusions sont précisées sur deux points. D'une part les néoblastes totipotents se trouvent répartis partout, jusqu'au voisinage de l'extrémité caudale; leur densité est telle que tout fragment même très petit, greffé dans la région antérieure, en contient assez pour, à la fois, réparer les lésions du territoire hôte et reconstituer une queue et une tête.

D'autre part la migration de ces néoblastes est déclenchée à partir d'une section passant en territoire prépharyngien, que celui-ci soit en place ou qu'il soit greffé en territoire postpharyngien.

Il y a lieu de souligner encore que la présence de néoblastes totipotents n'est pas suffisante pour assurer la régénération céphalique; il y faut aussi le territoire compétent, du moins dans le cadre de nos expériences de transplantations. Potentialité des néoblastes et potentialité des territoires sont deux notions

différentes même chez les planaires, deux notions qui ne se recouvrent pas nécessairement. La différence entre territoire à régénération totale et territoire à régénération limitée ne réside pas dans le matériel régénérateur mais dans le substrat; et celui-ci conserve ses propriétés lorsqu'il est transplanté.

RÉSUMÉ

1. Un greffon d'origine prépharyngienne, implanté en arrière de la bouche, tend à dominer le fragment postérieur obtenu après section postbuccale.

a) Si cette section passe dans le greffon, le bord libre de celui-ci régénère une tête; les bords de l'hôte viennent se fondre avec le régénérat.

b) Si le greffon ainsi sectionné a été irradié, la région post-pharyngienne lui fournit le matériel cellulaire utile à la régénération et le résultat obtenu est le même que précédemment, mis à part le fait que la régénération est retardée des quelque douze jours nécessaires à la migration des néoblastes sains dans le greffon irradié.

c) Si la section passe entre la bouche et le greffon, aucune régénération n'est possible. Le greffon se bombe dorsalement, acquiert des yeux et ses mouvements entraînent la partie postérieure.

2. Un greffon d'origine postpharyngienne, implanté à l'avant d'une planaire irradiée, fournit son matériel cellulaire sain à l'hôte, mais ne peut se régénérer lui-même.

SUMMARY

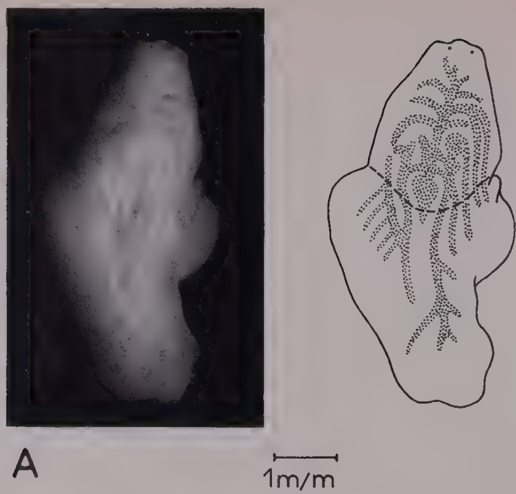
1. A graft of prepharyngeal origin, implanted behind the mouth, tends to dominate the posterior fragment obtained by postbuccal section.

(*a*) If this section runs through the graft, the latter regenerates a head at its free anterior border while the host edges undergo a regulation of form.

(*b*) After a similar section, the graft having previously been irradiated with X rays, the postpharyngeal host region provides the graft with the necessary material for regeneration and the final result is identical with the preceding one, except that regeneration is delayed for about twelve days during which the healthy neoblasts are migrating across the irradiated graft.

(*c*) If the section goes between mouth and graft, no regeneration is possible at the level of section. The graft bulges dorsally, develops eyes, and its movements draw the posterior part along.

2. A graft of postpharyngeal origin, implanted at the anterior level of a decapitated and X-irradiated worm, supplies the host with its own healthy cells, but it remains unable to perform any head regeneration from a free border running through itself.



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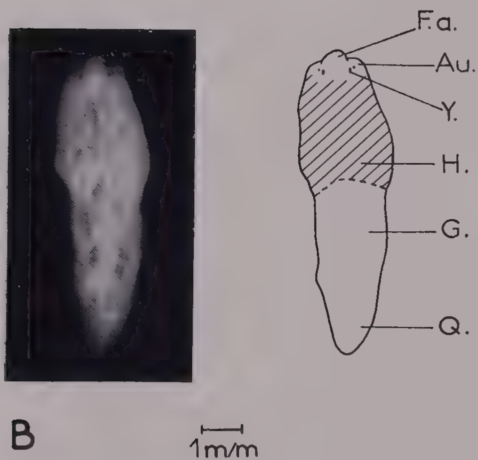

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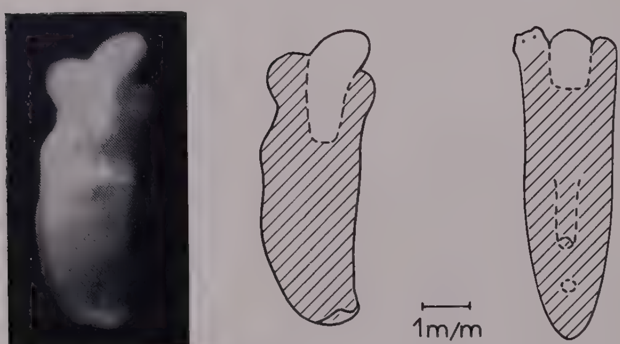

C A A′ B

F. STÉPHAN-DUBOIS *and* F. GILGENKRANTZ

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EXPLICATION DE LA PLANCHE

FIG. A. Même cas qu'en figure 3C dans le texte. Les limites intérieures du greffon étaient plus nettes sur l'animal vivant. Remarquer la jonction entre les diverticules digestifs des deux parties greffées.

FIG. B. Évolution du court fragment antérieur de l'expérience schématisée dans la fig. 5B dans le texte. Le petit greffon sain (*G.*) possède un stock suffisant de néoblastes pour régénérer une queue (*Q.*), pour réparer les radio-lésions de l'hôte (*H.*) et lui permettre sa régénération céphalique complète.

FIG. C. Évolution du long fragment postérieur de l'expérience schématisée dans la fig. 5B dans le texte. Le greffon ne se régénère jamais. Dans 10 cas (comme *A* et *A'*) les bords de l'hôte ne régénèrent pas. Dans 1 cas (*B*) un des bords régénère une tête complète et saine, les radio-lésions de l'hôte sont réparées par les néoblastes sains du greffon.

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A Study of Nuclei and Intercellular Ground Substance during the *in situ* Differentiation of Somites in *Taricha torosa*

by CYRIL V. FINNEGAN[1]

From the Department of Zoology University of British Columbia

WITH ONE PLATE

INTRODUCTION

In order better to evaluate results obtained in this laboratory concerning the responses of differentiating postneurula somite tissue to other mesoderm tissue placed in its immediate vicinity (Finnegan, unpublished), it was necessary to examine somite differentiation *in situ*. A qualitative examination of somite interphase nuclei of tail-bud and later stages was performed to note their morphological changes since it was assumed, as suggested by Briggs & King (1955), that such changes indicate cellular differentiation and, conversely, that absence of such changes indicates that the cells are not actively differentiating.

Because of the possible role of the intercellular matrix in histogenesis (see Grobstein, 1954, 1959; and Edds, 1958) a study was made of the development in the somite of that portion of the intercellular matrix which is demonstrable histochemically with the periodic acid-Schiff (PAS) technique. The visual clarity of the results has been materially aided by the fluorescent Schiff reagent of Culling & Vassar (1961) which makes possible a fluorescent Feulgen and a fluorescent PAS reaction.

EXPERIMENTAL PROCEDURES

Taricha torosa embryos from stage 25 up to 15 mm. in length (Twitty & Bodenstein stages; see Rugh, 1948) were fixed in Carnoy's acetic-alcohol mixture under standard conditions of temperature and time, paraffin processed, and sectioned at 10 μ. Sections were dried at 37° C. to avoid nucleic acid changes known to occur at high temperatures (Pearse, 1960). The acetic acid in this fixative precipitates nucleic acid (DNA) within the nuclear membrane. Despite the fact that this may produce a chromatin pattern artificial in appearance as compared to the living condition (Baker, 1950, 1958), a constant difference of

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results, produced by identical technical procedures, may be taken as indicative of some underlying phenomenon within the tested material (the 'good artifact' of Gersh, 1959).

Throughout the developmental stages examined the *somite* is considered as the block of mesoderm tissue lying lateral to the neural tube and notochord. The mesenchyme which appears in the sectioned material both medial and lateral to this block following stage 30 is not included.

The fluorescent Feulgen reaction was as described and discussed by Culling & Vassar (1961). A short acid hydrolysis (10 min.) was used to give the maximum staining response with the fixative (see Pearse, 1960). The intercellular material was demonstrated with the periodic acid-Schiff (PAS) reaction in which the fluorescent and conventional Schiff reagents were applied to the sections after a 10-minute exposure to the periodic acid and the sulphite rinses of the McManus procedure. Sections similarly stained after the Hotchkiss procedure for the conventional PAS technique, in which an acid-reducing rinse before exposure to periodic acid is substituted for the sulphite rinses, gave results identical with the above.

Both the Feulgen and the PAS reactions were controlled by (1) demonstrating the absence of preformed aldehydes (the omission of hydrochloric or periodic acid producing a negative response with the Schiff reagent); and (2) demonstrating that the aldehyde groups formed with the appropriate acid treatment could be blocked chemically from reacting with the Schiff reagent (acetylation gave a negative response in both reactions in the somite material). It appears, therefore, that the results obtained with these two histochemical reactions were due to the presence of an aldehyde produced by the action of the periodic or the hydrochloric acid rather than to any pre-existing aldehyde or reducing lipid.

Control of the Feulgen reaction was by treatment of sections with deoxyribonuclease and with perchloric acid prior to application of the Feulgen procedure; both of these pretreatments produced negative Feulgen reactions. Since a positive Feulgen reaction within nuclei is now generally accepted to be specific for DNA (Pollister & Ornstein, 1959; Pearse, 1960), the Feulgen-positive material observed in the sections will be so discussed in this report.

The specific determination of the PAS-positive intercellular material of the somites was attempted (1) by treatment with diastase to remove simple polysaccharides from the sections (the fluorescent PAS material remained); (2) by treatment with alcian blue, aldehyde fuchsin, and toluidine blue to demonstrate the presence of acid or sulphated mucopolysaccharides (all these agents gave negative results in the intercellular material under investigation); and (3) by staining with Sudan black B to locate any phospho- or glycolipids not previously removed by the fixing procedure (all sections tested gave negative results).

In view of the elimination of the presence of the potentially PAS-positive materials indicated by these tests it is assumed that the PAS-positive material described in this report is most probably neutral mucopolysaccharide.

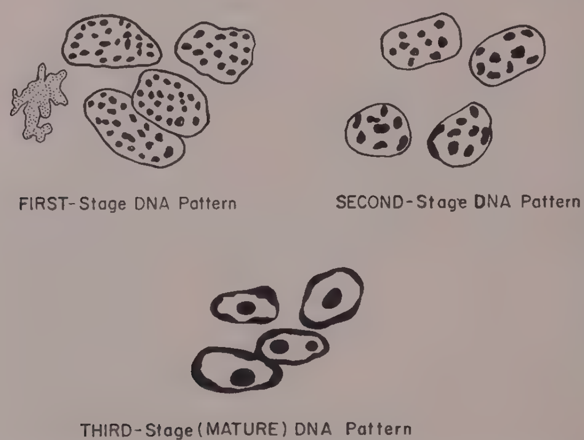
The fluorescent stained sections were observed and photographed with the Zeiss Large Fluorescence equipment as described by Vassar & Culling (1959).

EXPERIMENTAL RESULTS

Somite nuclear morphology

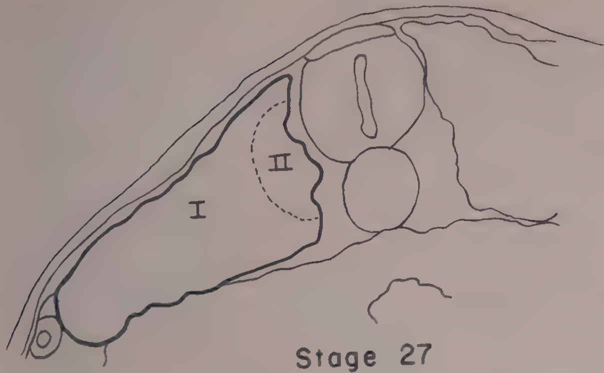
Stage 25–27 group

All somite nuclei in these embryonic stages demonstrated Feulgen-positive material (DNA) as fluorescent aggregates, rather evenly spread within the nuclear membrane; but those nuclei in cells of the medial area of the somite adjacent to the notochord-ventral neural tube region contained a coarser



TEXT-FIG. 1. The three varieties of chromatin (DNA) pattern observed in somite nuclei. For explanation see text. (Figures traced from photographs. Approx. ×500.)

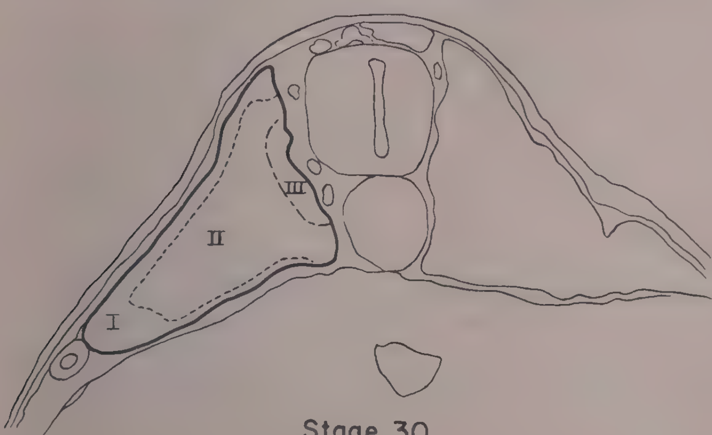
chromatin pattern and produced a less intense fluorescence than the rest (Plate, fig. F; see also Text-fig. 2). The more intense fluorescence in the lateral and middle somite nuclei was produced by a large number of individually small DNA aggregates within the nuclear membrane, to be referred to henceforth as the *first-stage chromatin pattern* (Text-fig. 1), while the more medial nuclei demonstrated a reduced number of individually larger DNA aggregates, the *second-stage chromatin pattern* (Text-fig. 1). Mitotic figures were encountered in the lateral and middle regions of the somite where the first-stage chromatin pattern was present in these developmental stages (Text-fig. 2) and may be observed in these areas in routine haematoxylin and eosin sections. Such figures are not encountered in the medial somite area where the second-stage chromatin pattern is observed (in Text-fig. 2, region of numeral II).



TEXT-FIG. 2. Outline of a section through an anterior trunk somite of a stage 27 *T. torosa* embryo. The numeral I indicates the location of the first-stage DNA pattern nuclei and the numeral II indicates the approximate location of second-stage nuclei in the somite. (Traced from a projection.)

Stage 28–30 group

The second-stage DNA pattern became visible in nuclei more laterally placed in the somite (Text-fig. 3), and in the oldest stages the most medial somite nuclei



TEXT-FIG. 3. Outline of a section through an anterior trunk somite of a stage 30 *T. torosa* embryo. Numerals I and II as in Text-fig. 2. Numeral III indicates the approximate location of the third-stage (mature) DNA pattern nuclei. (Traced from a projection.)

initiated a chromatin pattern in which the major portion of the DNA appeared to be coalesced near the nuclear membrane and the nucleoli. The nuclei with first-stage DNA pattern were present in the superficial areas of the somite; that is, in the dorsal tip, the lateral face, and the ventral tip regions (Text-fig. 3, numeral I). Again, mitotic figures were found only in these areas with the

first-stage chromatin pattern (Plate, fig. G) as also in haematoxylin and eosin sections. They were not encountered in other areas of the somite (Text-fig. 3, numeral II). The older animals in this group respond with a partial flexion of the trunk to strong stimulation with a hair loop.

Stage 33–36 group

The further coalescing of the Feulgen-positive material near the nuclear membrane and around the two nucleoli characteristic of this species (Costello & Henley, 1949) became more evident in the median somite nuclei of this developmental group. The coarse DNA aggregates of the second-stage pattern were no

Stage 36

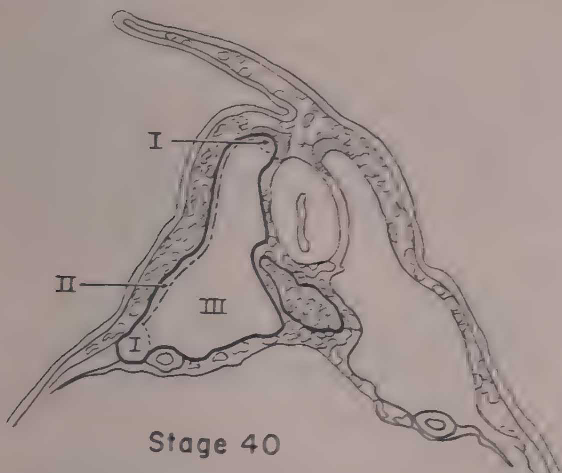
TEXT-FIG. 4. Outline of a section through an anterior trunk somite of a stage 36 *T. torosa* embryo. Numerals I, II, and III as in Text-fig. 3. (Traced from a projection.)

longer visible in these nuclei so that the nucleoli appeared suspended within the fluorescence-outlined nuclear membrane, the *third-stage* or *mature chromatin pattern* (Text-fig. 1). This condensation of the chromatin on the nucleoli and nuclear membrane has been noted in differentiating amphibian myoblasts by Sirlin & Elsdale (1959) and animals in this group independently perform C- and S-flexures. At this time only the most superficial layer of lateral-face nuclei and small groups of nuclei in the ventral and in the dorsal tip regions still showed the first-stage DNA pattern, while throughout the remaining portion of the somite nuclei with the second-stage DNA pattern were visible (Text-fig. 4).

Stage 40 group

The second-stage DNA pattern now appeared in the nuclei of the lateral face and the first-stage pattern remained only in the nuclei of the superficial dorsal and ventral tip area, the number of such nuclei (maximum of six per section) being greater in the ventral tip than in the dorsal tip. Elsewhere in the somite the

third-stage DNA pattern was present. It would seem, then, that in the *torosa* somite at stage 40 there exist two cell populations (growth centres) in which the first-stage or mitotically active DNA pattern is present, a small dorsal-tip group, and a larger ventral-tip group (Text-fig. 5). It is in these two areas that the occasional mitotic figure observed in somite tissue of these older animals is to be found.



TEXT-FIG. 5. Outline of a section through an anterior trunk somite of a stage 40 *T. torosa* embryo. Numerals I, II, and III as in Text-fig. 3. The dotted area overlying each of the dorsal somite tips represents the grouped melanophores which produce the dorsal band pigment pattern typical of this species. (Traced from a projection.)

14mm. larva group

The third-stage DNA pattern was present in nearly all somite nuclei at this time. The first-stage DNA pattern was no longer visible and the second-stage pattern appeared in a small group of nuclei (less than six per section) in the ventral tip. In the area immediately medial to the dorsal somite tip a small group of nuclei with the second-stage DNA pattern were present. This group may constitute a growth centre in *T. torosa* similar to the dorsal growth centre described by Holtzer & Detwiler (1953) for somites of *Ambystoma punctatum* though it must be noted that, as in the earlier developmental stages, mitotic figures were not observed in these regions occupied by cells with the second-stage chromatin pattern present.

Somite intercellular material

Stage 25–27 group

The intercellular PAS-positive material was discernible as a discontinuous granular system throughout the major portion of the somite. Adjacent to the notochord and neural tube the PAS-positive material was present as a

continuous entity which appeared to be physically continuous with the PAS-positive intercellular substance in the medial area of the somite. In some cases the reactive intercellular material of the medial area seems to be aligned with the intercellular material of the neural tube (Plate, fig. D). During these stages of development the superficial regions of the somite were free of, or contained small amounts of, the discontinuous granular PAS-positive intercellular material (Plate, fig. A).

Stage 28–30 group

The continuous PAS-positive substance now extended further peripherally from the medial area, the discontinuous granular material was evident in the dorsal tip and lateral face areas, and only the superficial ventral tip of the somite appeared to be free of the PAS-positive material.

Stage 33–36 group

The PAS-positive material became continuous throughout the somite during these stages except for the lateral face, the dorsal tip, and the ventral tip areas where the discontinuous granular phase was found (Plate, fig. C).

Stage 40 group

The now thicker continuous PAS-positive material covered the entire somite from the medial border to the lateral surface and appeared in the ventral and dorsal tip areas. However, the discontinuous granular stage constituted most of the PAS-positive substance present in the somite tip areas and the lateral surface region (Plate, fig. E).

14-mm. larva group

The entire somite section now demonstrated the presence of the continuous PAS-positive material. Accompanying it in the dorsal tip and ventral tip areas was a large amount of the discontinuous granular PAS-positive substance.

Somite reticulum

The basement membrane is considered to consist of a meshwork of argyrophilic reticular fibrils plus a homogeneous component or ground substance (Edds, 1958; Selby, 1959), the PAS-positive material being associated with the homogeneous ground substance. The appearance of this reticulum in the somite was examined with Gomori's silver impregnation method.

While argyrophilic fibres were demonstrable in the areas immediately external to the somite prior to stage 35 they did not appear intrasomitically until around stage 40, at which stage they were found only in the more medial portion and seemed to be continuous with the extrasomitic fibres of the axial area. Between stages 40 and 14 mm. reticular fibres became demonstrable throughout the

somite, though no argyrophilic material was found in the dorsal tip or in a small area in the ventral tip.

It would appear therefore that the reticular fibre component of the somite intercellular matrix is not demonstrable until some time after the thick continuous PAS-positive material is present between the somite cells. The fibres were initially demonstrable in the same medial region of the somite as, earlier in development, were the initial nuclear changes and the initial appearance of continuous PAS-positive material. Further, it would seem that the intercellular reticulum spreads laterally across the somite as did the changes in nuclear morphology and the PAS-positive intercellular material. At the oldest stages studied in this investigation (15 mm.) both the homogeneous ground substance and the reticular material were present in the intercellular spaces of the somite though it may be that, as suggested by Edds (1958), the ground substance is gradually replaced by the reticulum.

DISCUSSION

The results of the present investigation indicate that, as the *T. torosa* somite differentiates *in situ* following stage 25, there is present a population of mitotically active, presumably less differentiated, cells residing in the more superficial region of the somite, since up to stage 36 the entire superficial somite area demonstrates the presence of mitotic figures and the first-stage DNA pattern in the nuclei and the discontinuous PAS-positive material in the intercellular area (see Text-figs. 2–4). This population becomes more restricted spatially as the second- and third-stage DNA patterns with their apparent absence of mitotic figures and the continuous PAS-positive material spread peripherally until, at stage 40, there remain two small groups of cells in which the first-stage DNA pattern and the discontinuous PAS-positive material are present, in the dorsal tip and in the ventral tip of the somite (Text-fig. 5).

As measured by the techniques used, the medial area of the somite adjacent to the ventral neural tube–notochord area is further differentiated at any of the developmental stages examined than is the more superficial area, and phenomena associated with somite histogenesis (namely, nuclear chromatin pattern changes and the appearance of intercellular matrix) are initiated in this medial area of the somite appearing later further peripherally.

It has been suggested by Muchmore (1957, 1958) that neural, chordal, and other adjacent tissues influence myogenesis by confining somite tissue in a mass. Possibly this activity of neural and notochord tissue could take the form of initiating the production of somite intercellular ground substance, thereby producing a tissue mass within which histogenesis may occur. A further possible role of the PAS-positive material is indicated by the work of Heilbrunn (1952) and Heilbrunn *et al.* (1954) in which heparin-like substances appeared to have an inhibitory effect on cell-division. Thus, as the PAS-positive material appears

further laterad in the somite it may be inhibiting mitotic activity, thereby assisting differentiation. Finally, as demonstrated by Grobstein & Holtzer (1955; see also Holtzer, 1959) in cartilage production by mouse somite tissue, material of this axial intercellular matrix may be operative in an inductive manner.

SUMMARY

1. The differentiation of *T. torosa* somite mesoderm between stage 25 and 15 mm. has been examined with regard to variation in nuclear morphology, mitosis, and the development of intercellular matrix. Along with conventional techniques a fluorescent Schiff reagent has been used to obtain a fluorescent Feulgen for observation of DNA pattern, and a fluorescent PAS for observation of the appearance of intercellular mucopolysaccharide.

2. The development of three kinds of nuclear morphology (DNA pattern) and of the PAS-positive intercellular material of the differentiating somite is described.

3. The study indicates that nuclear changes and appearance of ground substance spread laterally from the always more differentiated medial portion of the somite and that mitotic activity is frequently observed in those more superficial areas containing cells with nuclei of the first-stage DNA pattern and is not (or is rarely) observed in those more medial areas containing cells with other DNA patterns and in which the PAS-positive intercellular material is present.

4. It is concluded that in the differentiating somite through stage 40 there exists a superficially located population of mitotically active, presumably undifferentiated, mesoderm cells.

RÉSUMÉ

Étude des noyaux et de la substance fondamentale intercellulaire au cours de la différenciation des somites in situ *chez le Triton* Taricha torosa

1. La différenciation du mésoderme somitique de *Taricha torosa*, entre le stade 25 et le stade 15 mm., a été étudiée sous le rapport des variations morphologiques des noyaux, de la mitose et de la formation de la matrice intercellulaire. Outre les techniques usuelles, on a utilisé un réactif de Schiff fluorescent afin d'obtenir une coloration de Feulgen fluorescente, pour observer la répartition de l'ADN, et un PAS fluorescent, pour observer l'apparition des mucopolysaccharides intercellulaires.

2. On décrit le développement de 3 types de morphologie nucléaire (répartition de l'ADN) et du matériel intercellulaire PAS-positif, dans le somite en cours de différenciation.

3. Ces recherches indiquent que les modifications nucléaires, et l'apparition de la substance fondamentale, s'étendent latéralement à partir de la partie médiane du somite, toujours plus différenciée; une activité mitotique s'observe fréquemment dans les zones plus superficielles, contenant des cellules à noyaux présentant

la répartition d'ADN caractéristique du premier stade, mais ne s'observe pas (ou seulement rarement) dans les zones plus centrales contenant des cellules dont les structures nucléaires sont différentes et dans lesquelles se trouve le matériel intercellulaire PAS-positif.

4. On conclut qu'il existe, dans le somite en différenciation jusqu'au stade 40, une population de cellules mésodermiques localisées superficiellement, à mitoses actives, et probablement indifférenciées.

ACKNOWLEDGEMENTS

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EXPLANATION OF PLATE

Fluorescent-Feulgen nuclei and Fluorescent-PAS-positive intercellular material during somite differentiation.

Fig. A. Stage 27. Section through an anterior trunk somite. The PAS-positive material of the neural tube (NT) and that of the medial face of the somite appear to be continuous in the area immediately dorsal to the notochord (NO). The discontinuous granular intercellular material is visible within the somite. Approx. $\times 500$.

Fig. B. Stage 28. The discontinuous granular PAS-positive intercellular material is apparent only in some regions of the lateral face of the somite. The overlying ectoderm (EC) is to the upper left. Compare with fig. E. Approx. $\times 500$.

Fig. C. Stage 36. A section through an anterior trunk somite. The continuous PAS-positive material, now apparent more laterad than at earlier stages, illustrates its appearance in older somites. The superficial lateral area, the dorsal tip, and the ventral area of the somite show the discontinuous granular PAS-positive stage in the original sections. Approx. $\times 100$.

Fig. D. Stage 27. A section through an anterior trunk somite. The PAS-positive material of the neural tube (NT) appears aligned with that of the medial part of the somite. The two tissues can be observed as separate entities in the original sections. Approx. $\times 120$.

Fig. E. Stage 40. The discontinuous granular PAS-positive material is now visible along the entire lateral face of the somite. The somite cells (to the right) show the intracellular glycogen displacement characteristic of fixed tissues and the ectodermal epithelium (to the left) demonstrates a thick basement membrane internally. Approx. $\times 500$.

Fig. F. Stage 25. A section through an anterior trunk somite with the medial face to the left side and the dorsal tip at the top of the photograph. The nuclei in the medial area (near notochord-ventral neural tube) are less fluorescent (second-stage DNA pattern) than are the more superficial nuclei (first-stage DNA pattern). Approx. $\times 120$.

Fig. G. Stage 28. A section through an anterior trunk somite with the medial face to the right side and the dorsal tip uppermost in the photograph. The second-stage DNA pattern nuclei in the medial area of the somite are less fluorescent than are the first-stage DNA pattern nuclei in the superficial (to the left) region of the somite. Note the mitotic figures in the areas of first-stage DNA pattern nuclei. Approx. $\times 120$.

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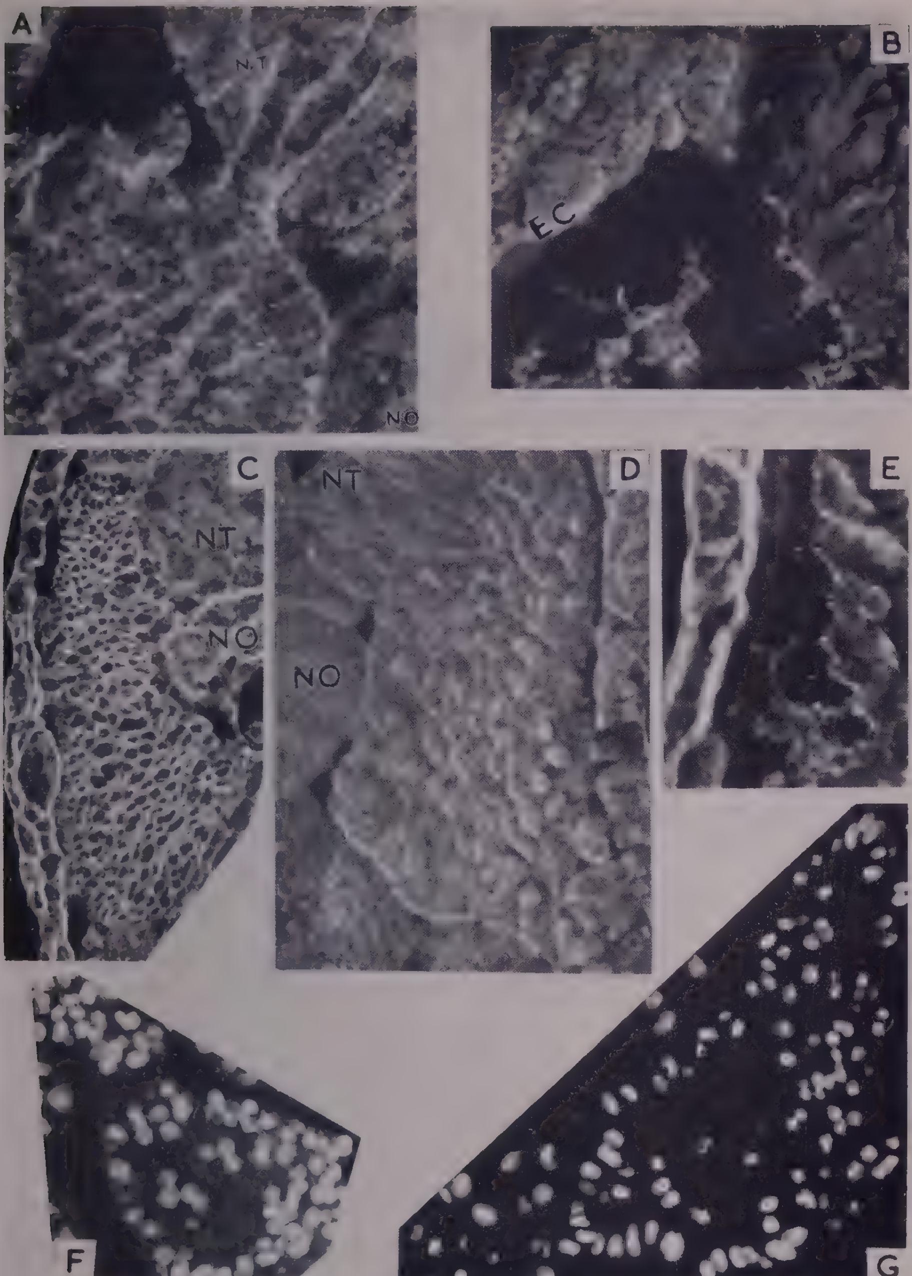

C. V. FINNEGAN

The Arrangement of Bristles in *Drosophila*[1]

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INTRODUCTION

MUCH of the geometrical complexity of animals and plants arises by the repetition of similar structures, often in a pattern which is constant for a species. In an earlier paper (Maynard Smith, 1960) some of the mechanisms whereby a constant number of structures in a linear series might arise were discussed. In this paper an attempt is made to extend the argument to cases where such structures are arranged in two-dimensional patterns on a surface, using the arrangement of bristles in *Drosophila* as illustrative material.

The bristles of *Drosophila* fall into two main classes, the microchaetes and the macrochaetes. A bristle of either type, together with its associated sensory nerve-cell, arises by the division of a single hypodermal cell. The macrochaetes are larger, and constant in number and position in a species, and in most cases throughout the family Drosophilidae. The microchaetes are smaller and more numerous, and show no fixed number or pattern in a species, although they do show some regularity in spacing. A number of mutants are known which alter the number and arrangement of the macrochaetes, usually by eliminating one or more pairs.

An explanation is put forward of the variations in the number and arrangement of the microchaetes, and of the macrochaetes in some mutant stocks, in terms of a common morphogenetic model. This model is an extension of that suggested by Stern (1956) in the light of mathematical considerations due to Turing (1952). It is hoped that this model may have some relevance to the arrangement of other repeated structures.

PREPATTERN AND COMPETENCE

Stern (1956) has analysed a number of bristle patterns in *Drosophila* by means of genetic mosaics. In the mutant achaete, which removes the posterior pair of dorsocentral bristles on the thorax, he showed that if in a predominantly wild-type fly a patch of genetically achaete tissue covered the site of the bristle, no bristle was formed; but if in a predominantly achaete fly the site of the bristle was covered by genetically wild-type tissue, a bristle was formed. He suggested

[1] This paper is dedicated to Professor L. C. Dunn in recognition of his long and distinguished career.

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that the presence of a bristle required the existence of a 'prepattern' determining its position, and the existence of cells competent to respond to the prepattern by forming a bristle. The normal prepattern exists both in wild-type and in achaete flies, but genetically achaete cells are incompetent to respond to this prepattern. An essentially similar conclusion was reached by Maynard Smith & Sondhi (1960) from a study of populations of *D. subobscura* homozygous for the mutant ocelli-less, which were selected for different expressions of the mutant.

The prepattern is most easily pictured as the distribution of an inducing substance with regions of high and low concentration, the regions of high concentration occurring at sites where bristles later form. A process whereby such a distribution could arise has been suggested by Turing (1952). He considered the distribution in a morphogenetic field of two chemical substances, or 'morphogens', together with an adequate supply of substrate from which they could be synthesized. These morphogens are free to diffuse and to react with each other. He showed that for certain values of the rates of reaction and diffusion the initial homogeneous equilibrium was unstable; any disturbance of the equilibrium, for example by Brownian movement, leads to the development of a standing wave of concentration of the morphogens. The actual pattern of peaks and valleys of concentration depends on the size and shape of the field, and on the 'chemical wavelength', i.e. the preferred spacing between peaks, which in turn depends on the rates of reaction and diffusion; it does not depend on the nature of the initial disturbance.

This provides a simple model of the process whereby a prepattern could arise. In cases in which a pattern is constant throughout a species, it is a more satisfactory one than Wigglesworth's competitive model (1959). According to Wigglesworth's model, the positions of the bristles will depend in part on which particular hypodermal cells happen by chance to be the first to differentiate, whereas Turing's mechanism would give rise to a pattern independent of the initial chance disturbance. Thus a competitive mechanism could explain the arrangement of a series of structures whose only regularity is the approximately equal spacing between them, but not of structures whose arrangement is constant from individual to individual.

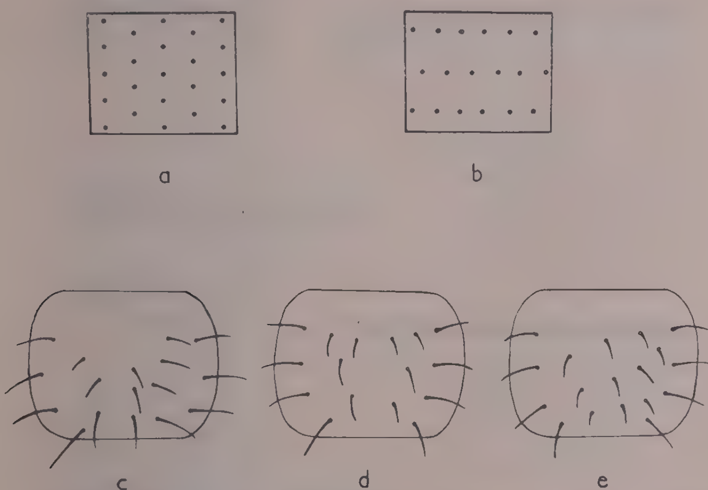
The way in which the arrangement of structures may depend on the shape of the field as a whole has been shown by Sengel's (1958) work on the development of feather papillae on the skin of the chick *in vitro*. If skin is removed from the dorsal region of the embryo when the feather rudiments have just become visible, these rudiments disappear, and later new feather papillae develop in different positions, the first to appear forming a row along the centre of the explant.

Turing's two-morphogen model is, however, too simple to explain all the facts. As Waddington has pointed out (1956), it would predict that different patterns would arise if particular stages of differentiation occurred in embryos of different sizes, and this is not always the case. But in spite of this and other difficulties,

Turing's suggestion of how a prepattern might arise is probably along the right lines.

THE ARRANGEMENT OF MICROCHAETES

For reasons of economy, it would be desirable to explain the arrangement of microchaetes and of macrochaetes by similar mechanisms, differing only in the accuracy with which they are regulated. Fortunately, there is some observational evidence that similar mechanisms are involved. In this section it will be shown that the arrangement of microchaetes does show traces of the kind of regularity to be expected if their positions depend on the shape of the field as a whole; in the next section it will be shown that at least some abnormalities of macrochaete arrangement are of the kind to be expected from variations of a prepattern arising in the way suggested by Turing.



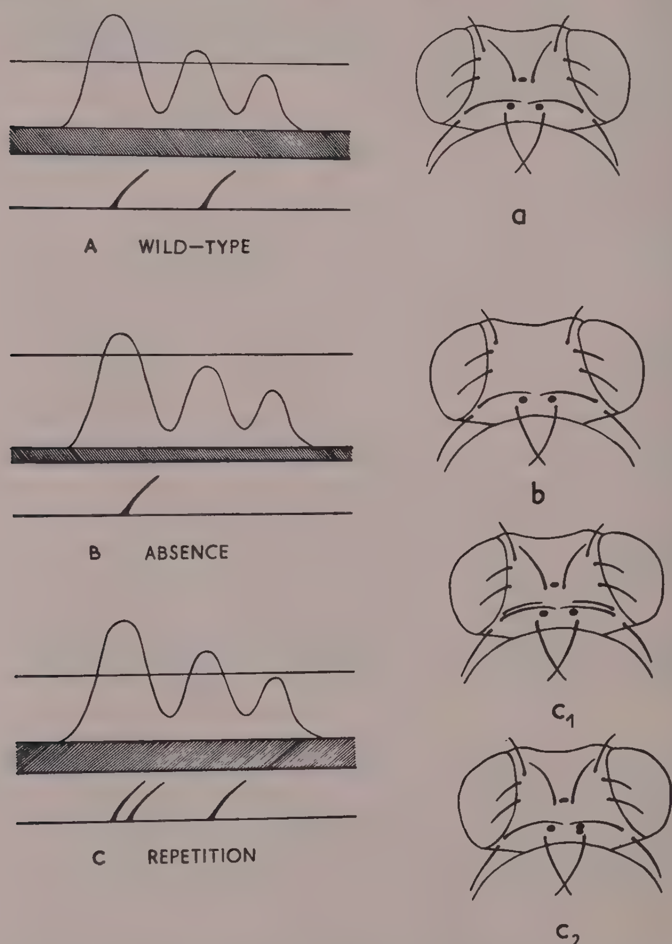
TEXT-FIG. 1. *a*, *b*, solutions of Turing's equations in two dimensions; the dots represent peaks of concentration of a morphogen. *c*, *d*, *e*, the arrangement of microchaetes on the fourth abdominal sternite in three individuals of *D. subobscura*.

Text-figs. 1 *a*, *b* show two solutions of Turing's equations in a uniform rectangular field, the dots corresponding to peaks of concentration of one of the morphogens; the solutions differ only because slightly different reaction rates have been assumed. Text-figs. 1 *c*, *d*, *e* show the arrangement of microchaetes on the sternites of the 4th abdominal segment of three individuals of *D. subobscura*. The first shows a somewhat irregular pattern, the second shows clear rows of bristles parallel to the boundaries of the sternite, and the third shows diagonal rows. The resemblance between the two latter sternites and the theoretical distributions is obvious. Sternites with these regular patterns are quite common in flies with low numbers of bristles, but irregular arrangements

are more usual in flies with larger numbers of bristles. Even an occasional sternite of the type shown in Text-fig. 1 *d* is sufficient to show that the pattern depends on the field as a whole, and such sternites are by no means uncommon.

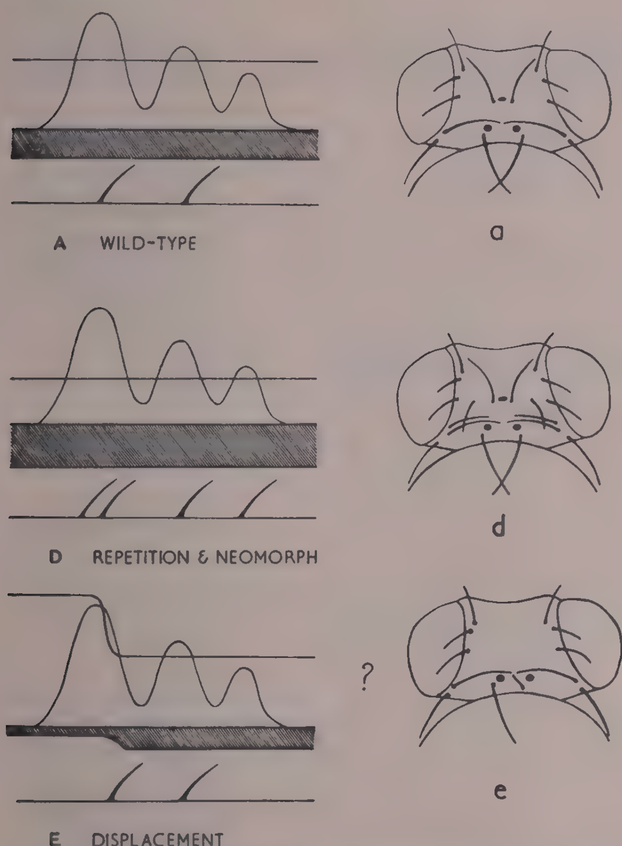
MACROCHAETES

Text-fig. 2 *a* shows the arrangement of macrochaetes and ocelli on the top of the head of *Drosophila*. The sex-linked recessive mutant ocelli-less in *D. subobscura* removes most of these structures. There is however, considerable variation in populations homozygous for the mutant, and by selective breeding for



Text-fig. 2. The arrangement of bristles and ocelli in *D. subobscura* in *a*, the wild-type, and *b*, c_1, c_2, individuals homozygous for the mutant ocelli-less. In the diagrams on the left, the curved lines represent the prepattern, pictured as a varying concentration of an inducing substance; the hatched areas represent the concentration of precursor; the upper horizontal lines represent the threshold level which the prepattern must reach if it is to induce a structure.

individuals with a larger or smaller number of structures, or with only particular structures present, a wide range of phenotypes, including the wild-type, have been obtained. These experiments have been described elsewhere (Maynard Smith & Sondhi, 1960; Sondhi, 1961 *a*, *b*, *c*). It has been shown that most of the results can be explained if it is assumed that there is an unvarying prepattern

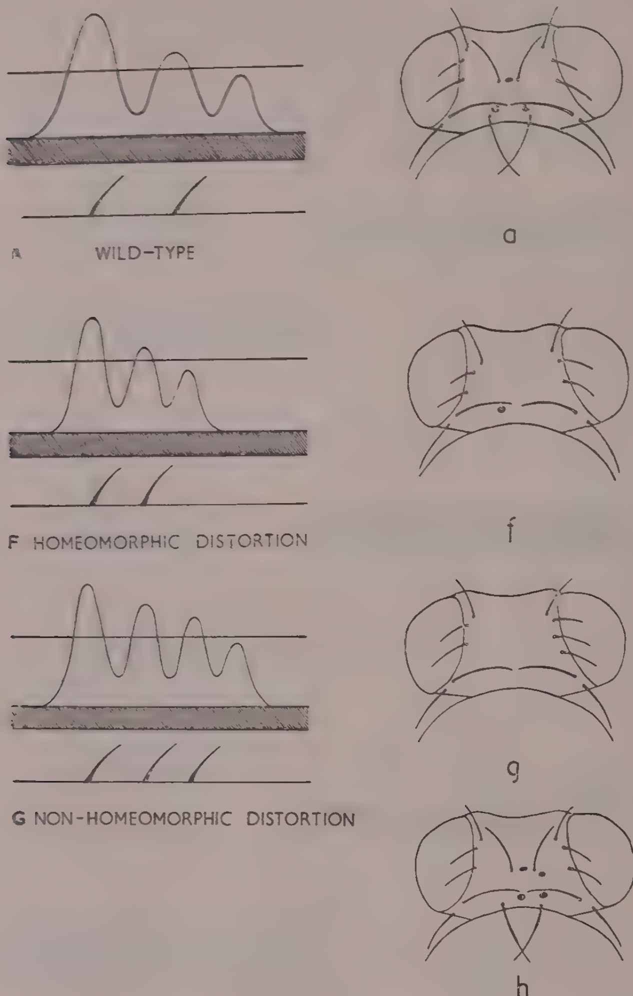


TEXT-FIG. 3. The arrangement of bristles and ocelli in *D. subobscura* in *a*, the wild-type, and *d*, *e*, individuals homozygous for the mutant ocelli-less. See legend to Text-fig. 2.

determining the positions of ocelli and bristles, and a varying amount of a common 'precursor' of ocelli and bristles. The absence of structures in unselected mutant stocks is due to the small amount of this precursor, but selection can both increase the amount of precursor, and also concentrate it in particular regions of the head. In this explanation the concept of 'amount of precursor' corresponds to Stern's concept of level of competence.

But, in addition to individuals lacking particular structures, we have also

obtained individuals with various other abnormal phenotypes. It will now be shown that these phenotypes can also be explained in terms of the prepattern-precursor model. Reference will be made to the populations in which these



TEXT-FIG. 4. The arrangement of bristles and ocelli in *D. subobscura* in *a*, the wild-type, and *f*, *g*, *h*, individuals homozygous for the mutant ocelli-less. See legend to Text-fig. 2.

phenotypes occur only when this is helpful in explaining their origin during development.

Text-figs. 2, 3, 4 show the various possible ways in which abnormal phenotypes can arise, and examples of phenotypes thought to have arisen in these ways. Text-figs. 2, 3 show changes arising from differences in the amount and

distribution of precursor, and Text-fig. 4 changes arising from alterations in the prepattern. The various possibilities are as follows.

Absence of structures, due to a low level of precursor (Text-fig. 2B). These are the typical abnormalities in unselected ocelli-less populations. Text-fig. 2 *b* shows the commonest phenotype in a population which had been selected so as to concentrate the precursor in the posterior region of the head and to remove it from the anterior region.

Repetition of structures (Text-fig. 2C). If the amount of precursor is greater than in the wild-type, it is possible that two structures should develop in response to a single peak of the prepattern. Text-figs. 2 c_1, c_2 show repetition of bristles and of ocelli respectively. Such repetition occurs mainly in populations which have been selected for an increased number of structures, and therefore presumably for an increased amount of precursor.

Neomorphs (Text-fig. 3D). If there can exist in mutant flies peaks of the prepattern to which genetically mutant tissue is incompetent to respond, it is possible that there also exist 'submerged' peaks of the prepattern to which wild-type tissue does not respond. If so, bristles might appear at the sites of these submerged peaks in mutant populations selected for increased competence. Text-fig. 3 *d* shows a phenotype thought to arise in this way. The new bristle occurs in about 5 per cent. of flies in a population which has been selected for many generations for an increased number of structures. It is confined to this population, and occurs only in individuals in which all the normal structures are present. The bristle is constant in its position and orientation, and closely resembles in these respects a bristle which occurs typically in flies of a related family (Sondhi, 1961*c*). The main reason for thinking that it arises by the process shown in Text-fig. 3D, and not from a change in the prepattern, is that its presence is not associated with any change in the positions of other bristles.

Displacement (Text-fig. 3E). If the precursor is absent at the peak of the prepattern, but present a short distance away, it is possible that a bristle would develop in a position slightly displaced from the normal. Stern (1956) has plausibly interpreted cases of displacement in his material in this way, but we cannot in our material always distinguish displacements arising in this way from those due to a distortion of the prepattern. This difficulty arises if a bristle is displaced without any associated displacement of neighbouring structures. This happens most commonly in the case of the postvertical bristles, particularly in populations selected for a low number of structures. The base of the bristle is displaced posteriorly and medially, and the displaced bristle is directed in an antero-medial direction (Text-fig. 3 *e*). Such displacements could be due to either of the mechanisms shown in Text-fig. 3E or 4F, although in the case of the postverticals the latter seems the more likely explanation, since the displacement is almost always in the same direction.

Homeomorphic distortion, i.e. a prepattern with the same number of peaks, but of a different shape (Text-fig. 4F). Text-fig. 4 *f* shows a phenotype thought to

arise in this way; it is common in populations selected for a low number of structures on the centre of the head. The displacement of a single bristle could also be due to the mechanism shown in Text-fig. 3E above, but in these flies all three orbital bristles are displaced posteriorly, and this could happen only if the prepattern itself is distorted.

Non-homeomorphic distortion, i.e. a prepattern with a different number of peaks from the wild-type (Text-fig. 4G). Text-fig. 4 *g* shows a phenotype thought to arise in this way; it also is common in populations selected for a low number of structures on the centre of the head. Four orbital bristles are present instead of three, and usually only the most anterior one is exactly in the position occupied by a bristle in the wild-type. The four bristles are evenly spaced; we have never observed the repetition of an orbital bristle, comparable to the repetition of a vertical bristle shown in Text-fig. 2 c_1. The interest of this particular phenotype is that it shows an unusual change in the arrangement of the macrochaetes, but one which arises by a mechanism which we believe to be that typically responsible for variations in the arrangement of microchaetes. In terms of Turing's model, the ratio between the chemical wavelength and the size of the field in which the waves are developing can vary within certain limits without involving any change in the actual pattern formed; but ultimately a threshold would be reached, involving the appearance of an additional bristle or bristles, and a respacing of other bristles in the field.

These mechanisms account satisfactorily for all the common abnormalities observed in ocelli-less populations. But occasionally there occur additional ocelli or bristles, at sites which are not occupied by such structures in the wild-type, and which vary from individual to individual in an irregular manner. Text-fig. 4 *h* shows such an additional ocellus. Additional ocelli and bristles of this sporadic type occur in populations selected for a high number of structures, with frequencies of about 0·9 per cent. and 0·6 per cent. respectively.

There is one unexpected feature of these results. It appears that a mutant, which was at first thought to affect only the amount and distribution of the precursor, also, although less commonly, modifies the prepattern, since the phenotypes in Text-fig. 4 *f*, *g* cannot easily be explained without this assumption. The mutant is therefore pleiotropic, in that it modifies two separate morphogenetic processes. But it seems plausible to suggest that the primary effect of the gene is to alter the concentration of precursor, and that this in turn may, in extreme cases, modify the development of the prepattern.

DISCUSSION

The development of specific structures at specific sites in *Drosophila* has been regarded as the result of two processes, one concerned with the formation of a prepattern which determines the positions at which structures are formed, and the other responsible for the competence of cells to respond to this prepattern by forming the appropriate structures. The justification of this division is that

the two processes can vary independently of one another. Our strongest evidence for thinking that the competence can vary while the prepattern remains unchanged comes from an ocelli-less population in which individuals were chosen as parents if they had the two posterior ocelli but lacked the anterior one (Maynard Smith & Sondhi, 1960). In this population the frequency of the selected phenotype increased from 15 to 64 per cent. At the same time, the frequency of individuals possessing the ocellar bristles, which lie close to the anterior ocellus, decreased almost to zero. In those individuals which did possess the anterior ocellus, the ocellus was not displaced posteriorly, but was usually much smaller than in the wild-type. These results only make sense if it is supposed that the prepattern determining the position of the anterior ocellus was unaffected by selection, and that the population changed because the competence of the cells to respond was reduced in the anterior part of the head. In contrast, our strongest reason for thinking that the prepattern can change are the phenotypes shown in Text-fig. 4 *f*, *g*.

The distinction between prepattern and competence is therefore made necessary by the nature of the variation observed. Variations in adult structure do not necessarily, or even usually, reflect changes in prepatterns. Differences between individuals may arise because of genetically determined differences in competence between their cells. The importance of this distinction has been increased by the work of Kroeger (1958) on the wing-hinges of *Ephestia*. He has been able to show that differences between serially homologous parts of the same individual may have a similar origin, in different responses of cells to identical prepatterns, although in this case the differences in response are not genetically determined, but arise in the course of embryonic differentiation. However, these successes in explaining variation in adult structure in terms of varying responses to unchanging prepatterns carry with them the danger that prepatterns may come to be regarded in a somewhat mystical light. It is therefore an important feature of the ocelli-less mutant that some of the abnormalities to which it gives rise can only be interpreted as the results of changes in the prepattern.

One question which it was hoped that this investigation would answer is whether the developmental mechanisms responsible for patterns which are constant in almost all members of a species have anything in common with those responsible for patterns which vary from individual to individual. The kinds of variation in the arrangements of microchaetes and of macrochaetes which have been described support the idea that the processes which determine the positions of the two types of bristle are similar. If so, the relative constancy of macrochaete patterns presumably arises because the number of macrochaetes in any particular pattern is small. It has been argued at length elsewhere (Maynard Smith, 1960) that mechanisms formally similar to that suggested by Turing can give rise to a constant pattern only if the number of peaks is small (approximately 5 to 7). The essence of the argument is that the number of

structures formed will be the nearest integer to the ratio of the size of the field to the 'chemical wavelength'; consequently the larger the number of structures which is to be kept constant, the smaller must be the coefficient of variation of this ratio. The simplest method of ensuring the constancy of larger numbers is by a process which was called 'multiplication'. The morphogenetic field is first divided by one patterning process into a small number of large regions, and then subdivided by a second process into a larger number of smaller regions. It is therefore interesting that Ursprung (1959) has shown that the development of the genital imaginal disks of *Drosophila* has a stepwise character of this kind. In more general terms, one reason for the stepwise nature of so many developmental processes may be that only processes of such a kind can give rise to uniform results.

If a number of structures are arranged in a linear series, as are for example a series of segments, a multiplicative process requires that two patterning processes be separated in time, occurring one after the other. But if the structures are arranged on a surface, another type of multiplicative process is possible, with an equivalent gain in accuracy. Two patterning processes can occur simultaneously, but along different axes; one process can determine the number of 'rows' and a second the number of structures in each row. This requires that the morphogenetic field should be initially anisotropic, whereas Turing supposed the field to be isotropic. It is therefore interesting that Weiss (1959) has shown that the regular arrangement of fish-scales depends on a pre-existing anisotropy; i.e. on the presence of two sets of collagen fibres at right angles to one another. In the cuticle of *Rhodnius*, on which the only structure visible on the adult tergites is a series of transverse ripples, Locke (1959) has demonstrated the presence both of an antero-posterior gradient and of a side-to-side polarity. The arrangement of microchaetes on the sternites of *Drosophila* suggests that the field is isotropic; compare the transverse rows, Text-fig. 1 *d*, with the diagonal rows, Text-fig. 1 *e*. But on the dorsal surface of the thorax there are reasons for supposing that the 'rows' and 'columns' are separately determined. There is a constant number of antero-posterior rows of microchaetes, the spacing between rows being appreciably greater than between bristles within a row. A connexion between the mechanisms responsible for the arrangement of macrochaetes and of microchaetes is also indicated, since the two pairs of dorsocentral macrochaetes always occur in the fifth row of microchaetes, counting from the mid-dorsal line, and each macrochaete occupies a position in the row which would otherwise be occupied by a microchaete.

SUMMARY

The bristles of *Drosophila* fall into two classes, the microchaetes which are small, numerous, and which vary in number from individual to individual, and the macrochaetes, which are larger, fewer in number, and constant in arrangement within a species, although many mutants which alter their number are

known. Variations in the arrangement of macrochaetes in populations of *D. subobscura* homozygous for the mutant ocelli-less are interpreted in terms of variations in a 'prepattern' determining the positions of the bristles, and of the competence of cells to respond to this prepattern by forming bristles. A process whereby such a prepattern may develop is described. It is argued that the arrangement of the microchaetes is determined by a similar process, differing only in that it is less accurately regulated. Mechanisms which may increase the accuracy of prepattern formation are discussed.

RÉSUMÉ

La disposition des soies chez la Drosophile

Les soies de *Drosophila* se répartissent en deux catégories. Les microchètes sont petites, nombreuses, et leur nombre varie d'un individu à l'autre. Les macrochètes sont plus grandes, moins nombreuses, et leur disposition est constante pour une espèce donnée, bien qu'on connaisse un grand nombre de mutations modifiant leur nombre. Des variations dans la disposition des macrochètes, chez des populations de *D. subobscura* homozygotes pour la mutation sans ocelles, sont interprétées en termes de variations d'un 'pré-arrangement' déterminant la position des soies, et de la compétence des cellules à réagir à ce 'pré-arrangement' en formant les soies. On décrit un processus selon lequel ce 'pré-arrangement' pourrait se réaliser. On soutient que la disposition des microchètes est déterminée par un processus similaire, en différant seulement par une régulation moins précise. On discute des mécanismes qui pourraient accroître la précision de la formation du 'pré-arrangement'.

ACKNOWLEDGEMENTS

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Comparison of the Different Teratogenic Effects of Three Commercial Samples of Trypan Blue

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INTRODUCTION

THE teratogenic action of the azo dye trypan blue (Gillman *et al.*, 1948) has been widely accepted. Commercially sold preparations of the dyestuff injected into pregnant rats at 8½ days of gestation usually cause a great increase in foetal mortality with subsequent intra-uterine resorption of the dead foetuses; at the same time a substantial proportion of the surviving foetuses are deformed. The efficacy of trypan blue preparations in causing malformations and foetal resorptions is dependent to some extent upon the genetic make-up of the animals used (Gunberg, 1958; Tuchmann-Duplessis & Mercier-Parot, 1959; Beck *et al.*, 1960). It is, therefore, necessary to use a susceptible pure-bred strain in experiments of this kind.

Other factors have also been shown to influence the teratogenic action of trypan blue. It was recently shown (Beck *et al.*, 1960) that the action of preparations of Grübler's trypan blue could be modified by recrystallization from hot water so that the resultant product caused significantly fewer foetal resorptions than did the parent compound. Preparations of the mother liquor remaining after the recrystallization were found to be as active as the parent compound in producing foetal resorption, and a preparation of the main coloured component of the mother liquor was found to produce a resorption rate which lay between the crude commercial product and the control group. No conclusion regarding the occurrence of malformations was advanced, since the M.R.C. hooded rats used in this experiment responded mainly by a raised resorption rate and produced very few malformed foetuses.

Furthermore, different commercially sold preparations of trypan blue have been found to produce quantitatively varied responses (Tuchmann-Duplessis & Mercier-Parot, 1959), but there must be some uncertainty as to the actual dose of trypan blue administered in these and similar experiments, since commercial samples of trypan blue contain highly variable amounts of sodium chloride, varying from 1·5 to 75 per cent.

The present investigation deals with the effects of three commercial preparations of trypan blue—standardized by titration to contain the same amount of

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azo dye—on a colony of Wistar rats which respond by producing both malformations and resorptions. Two preparations were found to be highly active but the third almost totally inactive. In addition, estimations of the Ld_{50} of one of the teratogenic and the non-teratogenic preparations were carried out on adult non-pregnant female rats in order to see whether there was any correlation between teratogenic activity and acute toxicity.

METHODS

The animals were divided into four groups. One group was injected with a preparation of trypan blue sold by G. T. Gurr, another with a preparation offered by Flatters & Garnett, and the third with an alternative preparation of Flatters & Garnett. The fourth was a control group. The dyes were given subcutaneously at $8\frac{1}{2}$ days of pregnancy in a dosage of 0·05 mol. of azo linkage/kg.; they were dissolved in normal saline to a concentration of 0·01 mol./ml. Controls received 5 ml. of normal saline/kg. The animals were killed at $20\frac{1}{2}$ days of pregnancy, the uteri were removed, resorption sites counted, and surviving foetuses examined for evidence of external malformation.

The concentration of azo grouping in each sample was estimated by volumetric titration against titanous chloride (British Pharmaceutical Codex, 1954) and this was repeated at the conclusion of the experiment to ensure that the sample had not deteriorated.

The acute toxicity (Ld_{50}) was calculated for the Gurr preparation and for batch 2 of the Flatters & Garnett preparation; Kärber's formula, which is designed for dealing with small series, was used (Kärber, 1931). Sixteen rats divided into 4 dosage groups of 4 rats per group were used in the Gurr series and 20 rats in 5 dosage groups of 4 rats per group in the Flatters & Garnett series. A single dose of 2 per cent. trypan blue in saline was given subcutaneously and death within 48 hours was considered to be a positive reaction. When a single injection was too bulky smaller quantities were injected simultaneously at various sites.

RESULTS

Table 1 is a summary of the results obtained. There is little difference between the response to injections of Gurr's trypan blue and that to injections of normal saline. No abnormal embryos were found and the slightly raised resorption rate following the injections of Gurr's trypan blue is not statistically significant ($0{\cdot}20 > P > 0{\cdot}10$).

Following the injection of the first sample of Flatters & Garnett trypan blue there were only 9 survivors from 33 implantation sites. Of these only 3 were normal and 6 were malformed. These values are significantly different from those obtained with the Gurr sample and also from the controls ($P < 0{\cdot}01$ in each case for resorptions, as well as for malformations when these are expressed as a proportion of the total survivors).

TABLE 1

Preparation	*Total implantations*	*Resorptions*	*Normal survivors*	*Abnormal survivors*	*No. of rats used*	*Ld₅₀ (Kärber)** $aM = D_m - \frac{\Sigma(z.d)}{m}$
Controls	56	2 (3·6%)	54 (96·4%)	0	6	—
G. T. Gurr	45	6 (13·3%)	39 (86·7%)	0	5	475 mgm./kg.
Flatters & Garnett (1)	33	24 (72·7%)†	3 (9·1%)†	6 (18·2%)†	5	—
Flatters & Garnett (2)	67	37 (55·2%)†	17 (25·4%)†	13 (19·4%)†	7	360 mgm./kg.

* aM arithmetical mean (Ld_{50}). D_m = dose at which all animals react. z = half the sum of reacting animals in two adjacent dosage groups. d difference in dose between two adjacent dosage groups. m = number of animals in each dosage group.

† All these values are significantly different from the control series ($P < 0{\cdot}01$).

The second sample obtained from Flatters & Garnett was also very active but less so than the first batch. However, the resorption and malformation rates are still significantly different from those of the Gurr and control groups ($P < 0{\cdot}01$ in each case). Out of 67 implantation sites there were 17 normal survivors only, 13 abnormal foetuses, and 37 resorptions.

Toxicity results are shown in the final column of Table 1. It is seen that the Ld_{50} of the Gurr sample was 475 mgm./kg. while that of batch 2 of Flatters & Garnett was only 360 mgm./kg. The significance of this difference is difficult to evaluate. If the standard error of each individual value is taken to be its square root, then the values of the Ld_{50} become 475 ± 44 mgm./kg. for the Gurr sample and 360 mgm. ± 38 mgm./kg. for the Flatters & Garnett sample (i.e. each value $\pm$ twice the standard error). The highest likely Ld_{50} for the Flatters & Garnett preparation is therefore 398 mgm./kg., and the lowest likely for the Gurr product 431 mgm./kg.; consequently there seems to be a real difference between the two figures. However, the calculation of the standard error in this case is open to criticism, and it is therefore impossible to state categorically that the difference in the toxicity of the two preparations was not due to chance.

DISCUSSION

The Gurr sample of trypan blue when tested for its teratogenicity is found to behave very differently from either of the Flatters & Garnett samples. Provided, therefore, that each sample is, in fact, trypan blue as the makers claim, the conclusion seems inescapable that commercially marketed trypan blue is not a pure dye. Indeed, preliminary chromatographic experiments at present being carried out in this laboratory show that all commercially marketed samples of trypan blue tested contain more than one chromogenic impurity. Commercial trypan blue is therefore a mixture of substances, and one, or a combination, of the components of the mixture may be teratogenic. It is, therefore, likely that the

differing potencies of the commercial samples used in this experiment depended upon the amount of teratogen(s) which each contained, and it is interesting to note that the non-teratogenic product appears to have been less toxic than the teratogenic sample tested. (The Ld_{50} of the third product could not be estimated since insufficient quantities were available.)

Whatever may be said about the purity of commercial samples of trypan blue, there is no doubt that the pure dye is theoretically a definite chemical entity to which a structural formula has been given (Conn, 1946). A method of preparation has been described (Hartwell & Fieser, 1936) and suggestions made for eliminating impurities which are bound to occur during the process of preparation of the dye. It seems unlikely that these methods of purification are being used in the commercial manufacture of the dye, and devices for preparation of the pure substance are at present under investigation in this laboratory. Whether trypan blue is itself a teratogenic agent must therefore remain an open question until it is tested in a completely pure form and can be distinguished from any possible closely related and similarly coloured contaminants.

SUMMARY

1. Three commercial preparations of trypan blue were injected into Wistar rats at 8½ days of pregnancy.

2. The amount of azo dye (in terms of titratable azo linkage) administered was the same in each series.

3. Two of the dye preparations were found to have well-marked teratogenic properties while the third was almost totally inactive.

4. Control rats were injected with normal saline.

5. One of the teratogenic dyes had a lower Ld_{50} (Kärber) than the non-teratogenic dye.

RÉSUMÉ

Comparaison entre les effets tératogènes différents exercés par trois échantillons commerciaux de bleu trypan

1. Trois préparations commerciales de bleu trypan ont été injectées à des rattes Wistar, à 8 j. ½ de gestation.

2. La quantité de colorant azoïque administrée (exprimée en liaisons azo titrables) a été la même pour chaque série.

3. Deux des préparations avaient des propriétés tératogènes bien marquées, tandis que la troisième était presque totalement inactive.

4. Une solution physiologique normale a été injectée à des rats témoins.

5. La dose létale 50 d'un des colorants tératogènes était plus faible que celle du colorant non tératogène.

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Le rôle du mesonephros de l'embryon de Poulet dans la nutrition de cellules cancéreuses

II. Etude par la méthode de la membrane vitelline

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AVEC SIX PLANCHES

NOUS avons montré que plusieurs souches cancéreuses humaines, HeLa, KB, Osgood, Fogh, peuvent être cultivées sur des organes embryonnaires de poulet explantés *in vitro*. Le mesonephros de 8 à 9 jours est un des plus favorables à la prolifération des cellules cancéreuses (Et. Wolff & Em. Wolff, 1958, 1959). Rappelons que celles-ci ne se multiplient pas et ne survivent pas, si elles sont placées directement sur les milieux nutritifs propres aux cultures organotypiques. Par contre, elles se nourrissent des tissus du mesonephros cultivés sur ces milieux. C'est donc l'organe embryonnaire, non le milieu de culture, qui est responsable du succès de la culture. Les cellules cancéreuses se comportent comme des parasites vis-à-vis des cellules de l'organe embryonnaire. Elles se substituent à elles, par un processus encore mal élucidé, car les cellules normales au milieu desquelles pénètrent les cellules cancéreuses ne montrent pas de phénomènes de dégénérescence. Pourtant elles disparaissent en grand nombre, car des territoires considérables du mesonephros sont envahis par les cellules tumorales, qui se substituent à elles et n'en laissent subsister aucune.

On peut se demander pour quelles raisons le mesonephros offre des conditions particulièrement favorables au développement des souches cancéreuses. Est-ce en raison de sa structure ou des substances nutritives qu'il élabore, qu'il est envahi par les cellules cancéreuses? En d'autres termes, des facteurs physiques, tels que la texture de l'organe, ou des facteurs chimiques, tels que des substances alimentaires, peuvent intervenir dans ce phénomène.

Le but du présent travail a été d'éprouver si un contact direct est nécessaire entre le mesonephros et les cellules cancéreuses pour que celles-ci prolifèrent activement. Qu'arrive-t-il, si l'on interpose une membrane anhiste entre les deux explants? Nous avons utilisé la membrane vitelline de l'œuf de poule, suivant la technique que nous avons décrite ailleurs et que nous rappellerons brièvement (Et. Wolff, 1960, 1961; Et. Wolff & Em. Wolff, 1960, 1961).

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Dans une première série d'expériences, nous avons associé des cellules cancéreuses à des explants de mesonephros à l'intérieur d'une pochette formée par un pli de la membrane vitelline. Ces recherches nous ont montré que cette membrane favorise beaucoup la dispersion et la pénétration des cellules cancéreuses à l'intérieur de l'explant.

Dans une deuxième série d'expériences, le mesonephros est cultivé seul dans un premier pli de la membrane vitelline, les cellules cancéreuses sont ensemencées dans un deuxième pli superposé au premier, de telle sorte qu'elles sont séparées du mesonephros par une épaisseur de membrane vitelline. Nous verrons que, dans cette situation, elles se multiplient encore très activement.

Dans une troisième série d'expériences, nous avons tenté de cultiver les cellules cancéreuses sur des extraits de mesonephros ou sur des milieux sur lesquels on avait explanté au préalable du mesonephros.

MATÉRIEL ET MÉTHODE

Nous employons comme milieu de culture un milieu dérivé du milieu standard de notre laboratoire (Et. Wolff & K. Haffen, 1952) dont la composition est la suivante:

gel d'agar à 1% préparé dans la solution de Gey	— 7 volumes,
extrait dilué d'embryons de poulet	— 3 volumes,
serum de cheval	— 3 volumes.

Un morceau de membrane vitelline d'œuf de poule non incubé est étalé sur le milieu de culture. De minces fragments de mesonephros sont placés les uns à côté des autres sur la membrane.

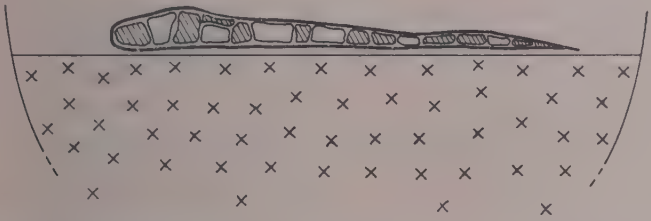

FIG. 1. Schéma de la culture d'explants dans une pochette de membrane vitelline, placée sur le milieu de culture. Les fragments de mesonephros et de cancer sont disposés en mosaïque dans la pochette.

1. Si on cultive les cellules cancéreuses au contact direct de l'organe embryonnaire, on dépose entre les explants ou sur les explants de mesonephros de petits amas de cellules cancéreuses. On replie ensuite un pan de la membrane sur les explants associés (fig. 1).

2. Si l'on désire interposer une membrane entre les deux types d'explants, on procède comme précédemment pour le mesonephros seul. On replie une première fois la membrane vitelline sur les explants de mesonephros.

On ensemence des groupes de cellules cancéreuses sur cette membrane, puis on la replie une deuxième fois. Ainsi les deux séries d'explants se trouvent chacune dans une poche de la membrane, séparée l'une de l'autre par une épaisseur de membrane (fig. 2).

Les cultures sont remises à la couveuse pour une durée de 5 à 7 jours. Elles peuvent être transférées, dans les mêmes conditions, sur un nouveau milieu.

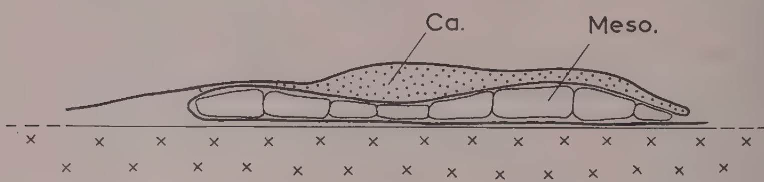

FIG. 2. Schéma du dispositif de la membrane repliée deux fois sur elle-même. A l'étage inférieur sont placés les explants du mesonephros (*Meso.*). A l'étage supérieur, les cellules cancéreuses (*Ca.*) forment des amas, séparés du mesonephros par une épaisseur de membrane.

RÉSULTATS

Associations directes de cellules cancéreuses et de mesonephros dans une même pochette

Nous avons fait des expériences sur le sarcome S 180 de souris, sur un chondrosarcome de souris, sur les souches humaines HeLa et KB.

La membrane vitelline favorise beaucoup la dissémination des cellules cancéreuses, qui rampent à sa surface, au contact des tissus du mesonephros (Planche 1, fig. 1). Les points de pénétration dans l'organe, à la faveur des lacunes, des fissures ou des tissus conjonctifs lâches, sont nombreux. Des plaques et des nodules se développent ainsi, qui envahissent progressivement le mesonephros en surface et en profondeur (Planche 1, fig. 2). Les différentes tumeurs conservent leurs caractères distinctifs. Les cellules du sarcome S 180 ont tendance à essaimer en s'insinuant individuellement entre les cellules conjonctives. Elles ont un aspect fuselé ou étoilé. Les cellules HeLa et KB forment des amas aux contours mieux délimités; serrées les unes entre les autres, elles ont un aspect dense, plus ou moins régulièrement géométrique, qui rappelle leur origine épithéliale (Planche 1, figs. 2 & 4). Un chondrosarcome originaire du laboratoire Fischer, de Copenhague, que nous entretenons sur des souris depuis plusieurs années, a été cultivé dans les mêmes conditions. Il est relativement peu envahissant et ne se propage pas loin de la place où il a été explanté. Mais ses cellules se multiplient très activement à l'intérieur ou au pourtour des foyers initiaux (Planche 1, fig. 3). De petites cellules indifférenciées, très proliférantes, se disposent au voisinage des tissus du mesonephros. Elles se divisent plusieurs fois de suite donnant naissance à de petits amas compacts de 4 à 24 cellules, rayonnant autour d'un centre. Ces amas, dont les cellules s'hypertrophient et finissent par dégénérer, s'encapsulent dans la substance fondamentale du cartilage. Des cellules jeunes, détachées

précocement de ces amas, continuent à proliférer, constituent de nouveaux massifs et propagent la tumeur à la ronde.

Il est très remarquable de suivre l'évolution d'une région du mesonephros en voie d'invasion. Les cellules tumorales s'insinuent dans le conjonctif et les lacunes situées entre les tubes urinifères, qu'elles enserrent. La paroi épithéliale de ces tubes s'amincit de plus en plus, ses cellules finissent par disparaître. Mais la lumière des tubes se maintient, elle est tapissée directement par les cellules cancéreuses. Dans le cas des souches épithéliales, les cellules se disposent régulièrement autour de ces canaux; bien qu'il ne reste plus rien des structures initiales, la masse tumorale est traversée des mêmes canalicules que le rein embryonnaire (Planche 1, fig. 4).

Interposition d'une membrane vitelline entre le mesonephros et les cellules cancéreuses

Dans le cas des souches cancéreuses humaines, on prélève avec une spatule de petits fragments du voile qui tapisse les flacons de culture. On les dispose sur la membrane vitelline qui recouvre les explants du mesonephros, en regard de ces explants. A titre de témoins, quelques groupes de cellules cancéreuses sont placés sur la membrane, dans une région où elle ne recouvre pas le mesonephros.

On opère d'une manière analogue avec le sarcome S 180. On découpe de petits morceaux d'un nodule tumoral sain, fraîchement prélevé sur une souris. On les dépose sur la membrane vitelline au-dessus des explants de mesonephros; d'autres sont placés, comme témoins, sur la membrane, au-dessus d'une région vide de mesonephros.

La membrane vitelline est composée de deux parties: l'une, d'aspect dense et homogène, de nature kératinoïde, enveloppe le jaune; l'autre, d'aspect fibreux, aux feuillets plus ou moins écartés, capable de se gonfler, constituée de mucine, est tournée vers l'albumine. Nous nous efforcions, dans les premières expériences, de placer les tissus à cultiver sur la face kératinisée (vitelline) de la membrane. Nous nous sommes aperçus par la suite que les deux faces peuvent être favorables à la culture des cellules cancéreuses, de telle sorte qu'il n'est pas toujours utile de donner à la membrane vitelline une orientation définie. Certaines variétés de cellules cancéreuses survivent et se propagent très bien dans les couches de mucine de la paroi externe.

Souches humaines d'origine épithéliale

Nous avons expérimenté sur les souches HeLa, KB et Fogh. Toutes trois montrent, à quelques différences près, le même comportement. Leurs cellules se multiplient activement, formant des amas importants, d'aspect lenticulaire. Renflés en leur centre, ils sont généralement amincis aux extrémités (Planche 2, figs. 5–7). Ils sont constitués de nombreuses couches de cellules. Le caractère le plus remarquable de telles cultures est qu'elles forment des nodules massifs, qui se développent dans les trois directions de l'espace, par opposition aux souches

cellulaires dont elles proviennent. De plus les cellules de ces cultures reconstituent des amas plus ou moins organisés, elles reprennent une structure épithéliale.

Dans la région centrale épaissie de l'amas, les cellules sont parfois groupées sans ordre. C'est aussi la région où, par suite d'une respiration défectueuse, certaines cellules se nécrosent. Les nodules les moins renflés, qui ne comportent pas plus de 6 à 10 assises de cellules, sont sains et prospères dans toute leur épaisseur. Les cellules qui se trouvent au voisinage des membranes vitellines ont une disposition très régulière. Celles qui sont situées contre la membrane inférieure, au voisinage du mesonephros, forment une ou plusieurs assises épithéliales à cellules hautes. Les cellules qui s'adossent à la membrane supérieure, qui les sépare de l'air, se disposent aussi en un épithélium, généralement moins haut que les assises épithéliales inférieures.

Les cellules de la souche KB, et surtout les cellules de la souche Fogh, se disposent souvent en épithéliums pluristratifiés, dont les assises forment un tissu palissadique très caractéristique (Planche 3, figs. 8, 9; Planche 4, fig. 10). Il convient de souligner que, dans ces conditions, les cellules tumorales retrouvent leur morphologie et leur organisation, qui n'apparaissent jamais dans les cultures histiotypiques en milieu liquide.

Après 5 à 7 jours de culture, les amas tumoraux se sont accrus considérablement. On peut suivre leur développement jour après jour en les observant au stéréomicroscope. L'étage des cellules cancéreuses se reconnaît nettement de l'étage du mesonephros, malgré la superposition et la transparence des deux sortes de tissus. Des variations d'éclairage permettent de distinguer les amas cancéreux qui apparaissent sous l'aspect de plaques réfringentes, charnues, piquetées de petits granules. Leurs bords sont généralement nets et incurvés (Planche 4, figs. 11, 12).

Ces massifs s'accroissent suivant les trois dimensions de l'espace: en épaisseur et en surface. La prolifération est particulièrement intense sur les bords des explants, et dans les couches qui adhèrent aux membranes. On voit souvent les cellules épithéliales se modeler intimement aux replis de la membrane, ou, par leur pression, la repousser vers le mesonephros, où elle forme des saillies et des denticules, qui s'enfoncent en coin dans les tissus nourriciers (Planche 2, fig. 7; Planche 3, figs. 8, 9).

Sur les bords latéraux des explants, les mitoses sont extrêmement nombreuses. Les nodules s'accroissent par la prolifération de leurs bords (Planche 5, fig. 14). Souvent des groupes de cellules se détachent de l'amas principal, ils forment de petits nodules secondaires, véritables métastases qui propagent la tumeur autour du foyer principal (Planche 4, fig. 13; Planche 5, fig. 15). Ces îlots sont constitués de cellules extrêmement proliférantes; parfois toutes sont simultanément en mitose.

De telles cultures peuvent être aisément transférées sur de nouveaux milieux. Il suffit de rabattre le pan de la membrane vitelline qui recouvre les explants cancéreux, de détacher ceux-ci de la membrane à laquelle ils sont adhérents, de

les découper en petits fragments. On ensemence alors ces morceaux sur une membrane vitelline recouvrant une culture de mesonephros, dans les mêmes conditions que dans la première expérience.

Les fragments de cancer, explantés comme témoins sur une partie de la membrane qui ne couvre pas le mesonephros, périclitent très rapidement. Ils sont en général complètement nécrosés après 5 jours de culture. Quand des cellules subsistent, elles sont très altérées, leurs noyaux sont anguleux, irréguliers, dilatés ou au contraire comprimés. Dans un cas unique, un explant de la souche KB, qui se montre dans toutes les expériences comme la plus résistante et la plus adaptable, présentait, à côté de cellules en mauvais état, des cellules saines et même quelques mitoses.

Ces expériences démontrent que les cellules des souches cancéreuses humaines peuvent vivre directement en parasites sur des explants de mesonephros de poulet, mais qu'elles peuvent aussi bien se nourrir des substances, élaborées par le mesonephros, qui passent par dialyse à travers une membrane. On remarquera que, dans le premier cas, les cellules du mesonephros sont détruites par les éléments cancéreux; dans le second cas, elles continuent à vivre et ne paraissent pas souffrir de la proximité des cellules cancéreuses.

Sarcome de souris S 180

Les fragments de sarcome, déposés sur la membrane vitelline au-dessus du mesonephros, donnent lieu à des migrations et à des proliférations intenses. Les cellules sarcomateuses ont tendance à déserter le centre des fragments, pour se presser contre les deux membranes, mais principalement contre la membrane inférieure qui les sépare du mesonephros. Elles forment, le long de cette membrane, une barrière de cellules très serrées, disposées très irrégulièrement, souvent en mitose. La forme de ces cellules est fuselée ou étoilée; leur aspect de grands fibroblastes ne diffère pas du type de la tumeur dont elles proviennent (Planche 6, figs. 16, 17).

Au pourtour des explants, les cellules du sarcome s'insinuent entre les deux feuillets de la membrane, elles forment une lame irrégulière, en général assez plate, avec des condensations plus ou moins volumineuses (Planche 6, fig. 16). Elles migrent parfois très loin de leur lieu d'origine, en se multipliant activement. Leur apparence est celle de fibroblastes en déplacement. Toutefois elles ne dépassent pas sensiblement les contours des explants de mesonephros, faute d'aliment.

Un cas particulier illustre l'attraction qu'ont les cellules sarcomateuses pour le mesonephros. Nous avons traité, dans certaines expériences, la membrane vitelline par la trypsine pendant 2 à 10 minutes. Puis nous avons procédé comme d'habitude, la membrane vitelline trypsinée servant de barrière entre les explants de mesonephros et ceux du sarcome. Ainsi traitée, la couche interne disparaît, la couche externe se gonfle et devient visqueuse. Tout en faisant encore barrage entre les deux explants, la membrane offre cependant moins de résistance au

passage des cellules; elle peut être transgressée en certains points. On voit alors les cellules sarcomateuses se presser à travers les brèches et s'approcher des cellules du mesonephros. Nous n'avons pas observé d'invasion proprement dite, bien que les cellules tumorales aient progressé en de rares points jusqu'au contact des tubes urinaires (Planche 6, fig. 18). Mais on voit, à la faveur de ces brèches, une masse de cellules faire irruption à travers la membrane en direction du mesonephros. Toutes ces cellules ont l'aspect de fibroblastes en mouvement, beaucoup d'entre elles sont en division. Ces sortes d'éruption mettent en évidence l'attraction exercée par le mesonephros, à la faveur des substances nutritives qu'il sécrète.

Action d'extraits de mesonephros ou de milieux 'conditionnés' par le mesonephros

Il résulte de la série précédente d'expériences que le mesonephros élabore des substances diffusibles ou dialysables, qui assurent la nutrition de certaines tumeurs. Nous avons cherché à mettre en évidence ces substances dans des extraits de mesonephros, après broyage et centrifugation.

Extraits de mesonephros

60 à 100 mesonephros d'embryons de 8 jours $\frac{1}{2}$ sont finement broyés au microboyeur. L'homogénat, additionné de quelques gouttes de liquide de Tyrode, est centrifugé à 16000 tours/minute pendant 10 minutes à la température de 0° C. On utilise le surnageant comme extrait brut. Les fragments de sarcome ou les colonies de cellules cancéreuses humaines sont ensemencés dans une pochette de membrane vitelline. Une goutte d'extrait est déposée sur la face supérieure de la pochette le premier jour de la culture. Une 2e, éventuellement une 3e goutte est ajoutée à intervalles de 1, 2 ou 3 jours. Les explants sont fixés et étudiés après 5 à 7 jours de culture.

Les cultures de KB se développent très bien dans ces conditions. Elles forment des amas prospères qui se multiplient activement dans 14 cas sur 15 cas. On voit de très nombreuses cellules se déplacer le long de la membrane vitelline, à laquelle elles adhèrent en formant une couche épithéliale saine et très prolifique. Avec les souches HeLa, les résultats sont moins bons. Dans deux cultures, les cellules HeLa offrent un aspect sain, forment des amas lenticulaires bien constitués, se multiplient modérément. Leurs migrations sont très faibles. Dans deux autres cas, les groupes cellulaires ensemencés ne se développent pas; à côté d'éléments sains, il y a beaucoup de cellules en nécrose; on ne trouve aucune figure mitotique. Dans le cas du sarcome S 180, les résultats ont été entièrement négatifs: les fragments de tumeur se sont complètement nécrosés, sans avoir donné naissance à aucune migration, ni à aucune prolifération.

Il est possible que l'extrait employé contienne quelque substance toxique,. à laquelle certaines tumeurs seraient plus sensibles que d'autres. Un précipité se sédimente sur les membranes, à l'emplacement où les gouttes de surnageant ont

été versées: ce dépôt peut aussi être préjudiciable à la respiration des tissus. Les cellules KB s'adaptent à ces conditions, le sarcome S 180 ne peut les supporter, les cellules HeLa montrent une tolérance relative.

Encore que la souche KB s'avère mieux adaptable que d'autres, elle donne cependant la preuve qu'il y a réellement dans l'extrait de mesonephros des substances capables de nourrir ou de stimuler des cellules malignes.

Milieux 'conditionnés' au mesonephros

Pour éviter la toxicité probable des extraits bruts de mesonephros vis-à-vis de la tumeur S 180, nous nous sommes adressés à la technique des milieux 'conditionnés'.

Nous explantons pendant 3 ou 4 jours des morceaux de mesonephros sur le milieu habituel de culture. Après avoir repéré exactement l'emplacement de ces explants sur le milieu, on les enlève avec précaution. Les fragments de mesonephros se sont soudés entre eux en une sorte de gâteau plat; ils se détachent en général tous ensemble. On dispose alors, à cette place, des fragments frais d'une tumeur prélevée sur une souris. On les recouvre d'une membrane vitelline, afin que les cellules puissent y adhérer et migrer. Dans tous les cas, les explants prolifèrent et donnent naissance à d'importantes migrations. Les cellules du sarcome se répandent très loin le long de la membrane, en couches généralement assez minces, mais denses. Elles ont un aspect sain et montrent de nombreuses figures de division. En définitive, ce procédé remédie à l'inconvénient des extraits obtenus par broyage. Il montre que les substances indispensables à la prolifération des cellules cancéreuses sont libérées dans le milieu, et conservent leur activité en l'absence du mesonephros qui les a produites. Il n'est pas douteux qu'en purifiant l'extrait brut de mesonephros on obtiendrait le même résultat.

CONCLUSIONS

Des cellules cancéreuses de différents types — originaires de tumeurs de souris ou d'hommes — peuvent se nourrir de deux manières aux dépens d'explants de mesonephros de poulet, cultivés *in vitro*. Elles peuvent se comporter en parasites des cellules embryonnaires, qu'elles détruisent et qu'elles remplacent. Elles peuvent aussi se nourrir des substances qui filtrent à travers une membrane dialysante, sans dommage apparent pour les tissus du mesonephros. La membrane vitelline du jaune d'œuf de poule permet d'éprouver ces deux modalités, soit en fournissant une enveloppe commune où se mêlent les deux tissus, soit en interposant un filtre entre eux. A la question posée dans notre introduction — le mesonephros agit-il par sa structure physique ou par des sécrétions chimiques? — nous pouvons maintenant répondre. Mais le problème admet deux solutions. C'est en vertu de son organisation interne que le mesonephros ouvre, plus qu'aucun autre organe embryonnaire, des voies à l'invasion cancéreuse. Il faut en rendre responsable sa structure lacunaire, aérée, sa richesse en tissu conjonctif, les chemins tracés par les membranes basales des canalicules. Mais c'est aussi en

raison de son chimisme propre, des substances qu'il élabore, qu'il offre un milieu favorable à la nutrition du cancer.

Ces deux solutions ne sont-elles pas contradictoires? Comment admettre que, dans un cas, le mesonephros soit détruit par les cellules qui l'envahissent, et que, dans l'autre, il puisse les nourrir à distance, sans subir de dommage? Nous devons admettre ces résultats, sans pouvoir encore les concilier. Il est possible que, dans l'invasion de tissus sains par les cellules cancéreuses, celles-ci se nourrissent d'abord des produits élaborés par ceux-là. Les destructions seraient secondaires, elles proviendraient d'une asphyxie, ou simplement d'une compression mécanique due à l'extraordinaire pullulation des cellules tumorales. Il est encore possible que les cellules cancéreuses élaborent des substances, par exemple des enzymes, qui activent les sécrétions des cellules saines, et qui en même temps intoxiquent celles-ci. D'autres hypothèses peuvent encore être envisagées qui touchent à l'important problème du mode d'attaque des cellules cancéreuses. Nous apportons à ce problème deux solutions qui sont valables dans les conditions de deux modalités différentes d'expériences. Mais nous ne prétendons pas expliquer les causes générales de l'invasion d'un organe par une tumeur.

Ayant démontré que des substances favorables à la prolifération cancéreuse sont élaborées par le mesonephros et qu'elles sont capables de passer à travers une membrane dialysante, nous pouvons nous demander quelle est la nature de ces substances. Il est certain qu'elles sont indispensables à la nutrition des cellules cancéreuses, dans les conditions de nos expériences. Apportent-elles *toutes les substances* alimentaires nécessaires, ou seulement des facteurs indispensables à l'utilisation des constituants du milieu. On peut supposer en effet que ceux-ci sont utilisés par les cellules cancéreuses, en la présence nécessaire d'une substance élaborée par le mesonephros. On peut aussi supposer que la membrane vitelline non seulement laisse filtrer des composés nutritifs, mais qu'elle puisse fournir par elle-même un appoint au régime alimentaire des explants: encore ne peut-elle le faire qu'en la présence de ce même facteur originaire du mesonephros.

Il n'est pas possible actuellement de résoudre cette question. La culture directe de cellules cancéreuses sur des explants de mesonephros fait penser qu'elles vivent exclusivement en parasites sur ces tissus qu'elles envahissent et détruisent. D'autre part, des explants de mesonephros ont été cultivés sur des milieux synthétiques relativement pauvres (par exemple le milieu 199 de Parker). Ils sont enlevés après 3 jours; à leur emplacement on place une membrane vitelline sous laquelle sont déposées des cellules KB. Dans cette expérience, le milieu est imprégné des substances élaborées par le mesonephros, comme dans les expériences en milieux naturels. Cependant la prolifération des cellules malignes est beaucoup moins bonne que dans les cultures sur milieux naturels. Cela pourrait indiquer que les explants tumoraux utilisent des substances nutritives de tels milieux, avec l'appoint obligatoire des facteurs du mesonephros. Mais cela

peut aussi signifier que le mesonephros, moins bien nourri sur un milieu synthétique, n'a pas diffusé en quantité suffisante les substances favorables aux tumeurs. La question n'est donc pas encore résolue. S'agit-il d'un 'aliment total' ou d'une substance stimulatrice? Dans le premier cas, l'aliment est-il uniquement formé de substances banales, telles que des acides aminés ou des peptides courants? ou l'une d'elles est-elle nettement spécifique? Dans le deuxième cas, le même problème se poserait: la substance stimulatrice est-elle un catalyseur banal, un enzyme très répandu, ou s'agit-il d'un composé spécifique, qu'on rencontrerait de préférence dans certains organes de l'embryon de poulet?

Ces questions que nos recherches permettent de poser et peut-être d'aborder par la voie expérimentale sont de nature à nous éclairer sur la nutrition et la propagation des cellules cancéreuses dans un milieu tissulaire et non dans des milieux artificiels, où certaines d'entre elles sont alimentées depuis des années et ont peut-être perdu leurs exigences spécifiques.

RÉSUMÉ

1. La membrane vitelline de l'œuf de poule a été employée comme adjuvant de la culture de cellules cancéreuses sur organes embryonnaires de poulet.

2. On enveloppe, dans une telle membrane, des fragments de mesonephros de poulet et de cellules cancéreuses préalablement associés. La pochette qui contient une telle mosaïque est placée sur les milieux de culture, à base d'agar, d'extrait d'embryon, de serum de poulain. La membrane inférieure permet les échanges avec le milieu nutritif, la membrane supérieure avec l'atmosphère (fig. 1).

3. Les cellules cancéreuses se répandent et se déplacent en grand nombre le long de la membrane, elles envahissent le mesonephros en de nombreux points. On obtient ainsi des cultures extrêmement prospères de différentes tumeurs de souris (sarcome, chondrosarcome) ou de souches humaines (HeLa, KB, Fogh) (Planche 1, figs. 1–4).

4. On emploie la membrane vitelline comme membrane de dialyse entre le mesonephros et les cellules cancéreuses (fig. 2). Celles-ci, qui ne peuvent se nourrir directement des aliments du milieu, tirent parti des substances élaborées par le mesonephros qui passent à travers la membrane. Des colonies de cellules cancéreuses très abondantes et très proliférantes prennent ainsi naissance. Elles croissent dans les trois directions de l'espace, sous forme de nodules massifs, plus ou moins organisés (Planche 2, figs. 5–7).

5. Les cellules d'origine épithéliale des souches humaines donnent naissance à des formations épithéliales caractéristiques, ayant parfois l'aspect palissadique (Planches 3 et 4). Les cellules du sarcome de souris S 180 conservent leur aspect incoordonné, leur tendance à la migration individuelle, leur forme étirée ou étoilée de grands fibroblastes (Planche 6).

6. Les colonies primaires des différentes catégories de tumeurs s'accroissent par la prolifération et l'essaimage des cellules de bordure, qui forment des nodules

secondaires et propagent la tumeur jusqu'aux limites de la région sus-jacente au mesonephros (Planches 4 et 5).

7. Le surnageant de l'extrait centrifugé du mesonephros conserve ses propriétés nutritives ou stimulantes vis-à-vis des cellules KB et HeLa. Il n'est pas favorable au sarcome S 180, probablement en raison de sa toxicité. Mais la culture sur un milieu 'conditionné', dans lequel a été cultivé préalablement du mesonephros, permet au sarcome S 180 de se multiplier et de se propager activement. La sécrétion du mesonephros, en l'absence de cet organe, suffit à assurer la survie et la croissance de la tumeur.

SUMMARY

1. The vitelline membrane of the hen's egg has been used as an aid to the culture of cancer cells on organs of the chick embryo.

2. Fragments of chick mesonephros and cancer cells are placed in association and then wrapped in the membrane. The pocket of membrane containing the association is placed on a culture medium consisting of agar, embryo extract, and horse serum. The lower membrane allows exchange with the nutrient medium, the upper with the atmosphere (Text-fig. 1).

3. The cancer cells spread and migrate in large numbers along the membrane, invading the mesonephros at many points. Highly prosperous cultures were thus obtained of mouse tumours (sarcoma, chondrosarcoma) or of human strains (HeLa, KB, Fogh) (Plate 1, figs. 1–4).

4. The vitelline membrane was used as a dialysis membrane between the mesonephros and the cancer cells (Text-fig. 2). The latter, which were unable to obtain nourishment directly from the medium, used substances elaborated by the mesonephros, which traversed the membrane. Colonies of abundant cancer cells, actively proliferating, thus arose. They grew in three dimensions, in the form of massive nodules, more or less organized (Plate 2, figs. 5–7).

5. Cells of epithelial origin of the human strains produced characteristic epithelial formations, at times with a palisade appearance (Plates 3 and 4). Mouse sarcoma cells (S 180) kept their disorderly arrangement, their tendency to migrate individually, and the extended or branched shape of large fibroblasts (Plate 6).

6. Primary colonies of the different kinds of tumour grew by proliferation, and by the detachment of groups of cells from the edge which form secondary nodules and spread the tumour to the limits of the region overlying the mesonephros (Plates 4 and 5).

7. The supernatant of a centrifuged extract of mesonephros keeps its nutritive or stimulating properties towards KB or HeLa cells. It was unfavourable for sarcoma S 180, probably because of its toxicity. But culture on a 'conditioned' medium, in which mesonephros had previously been cultivated, allows sarcoma S 180 to multiply and propagate actively. The secretion of the mesonephros, in

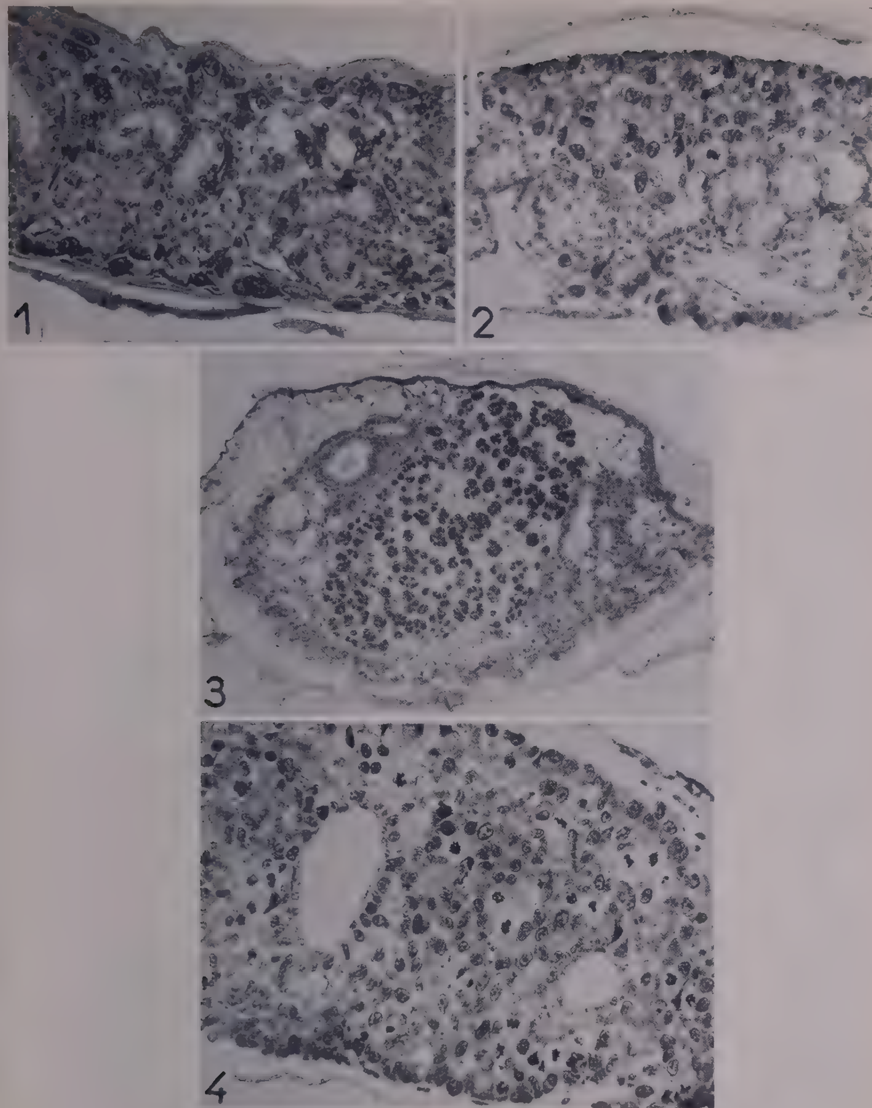

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Planche 1

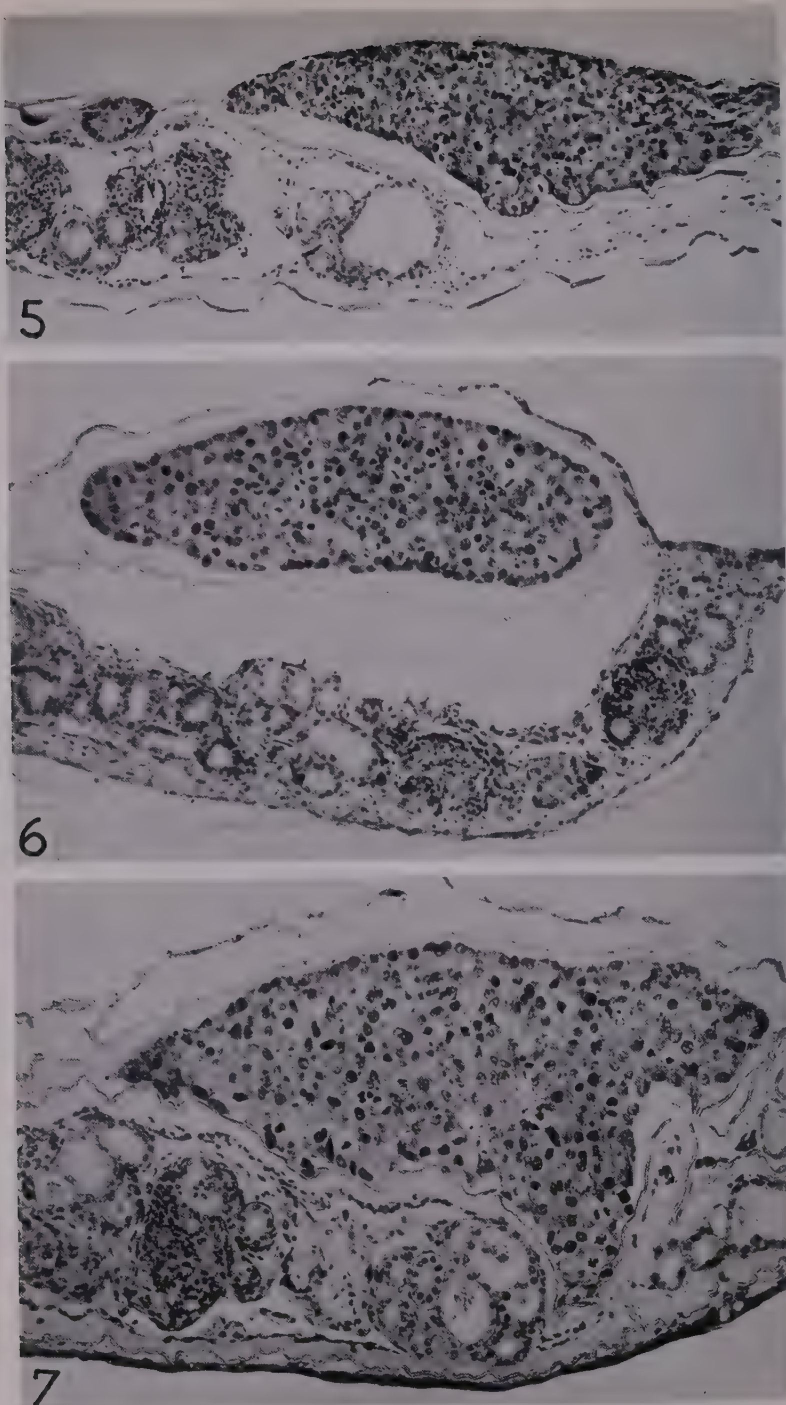

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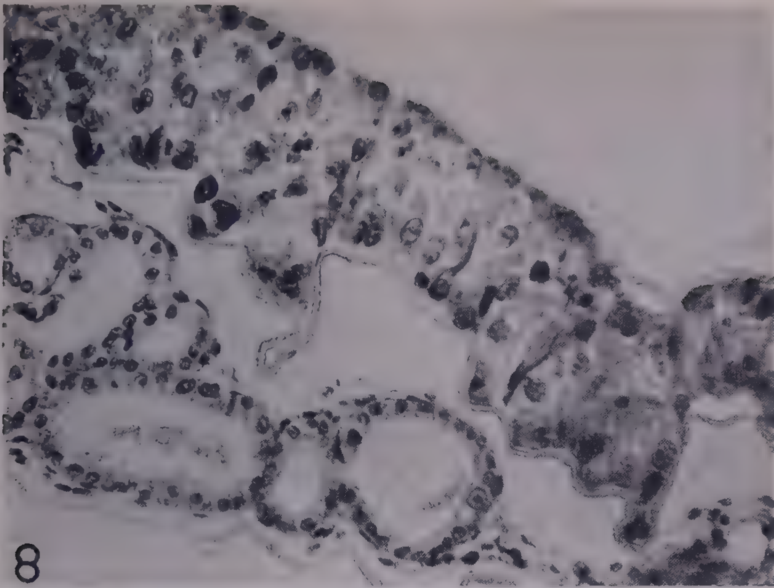
Planche 2



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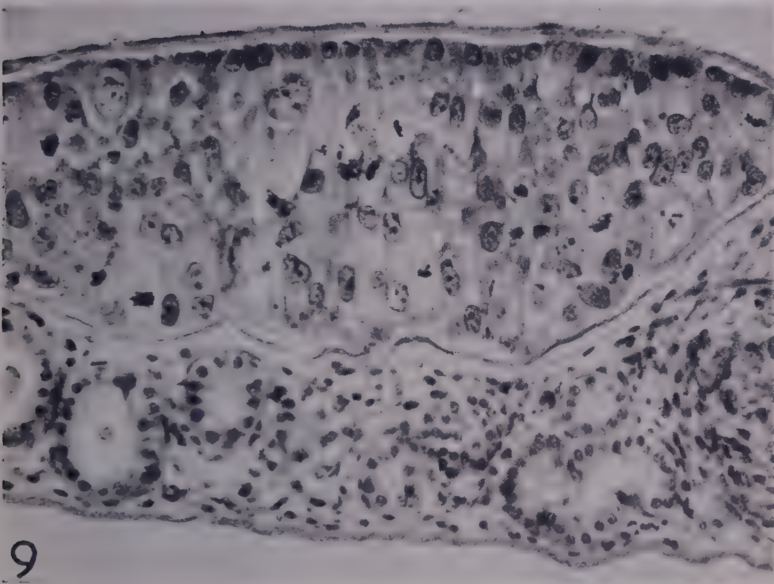

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Planche 3

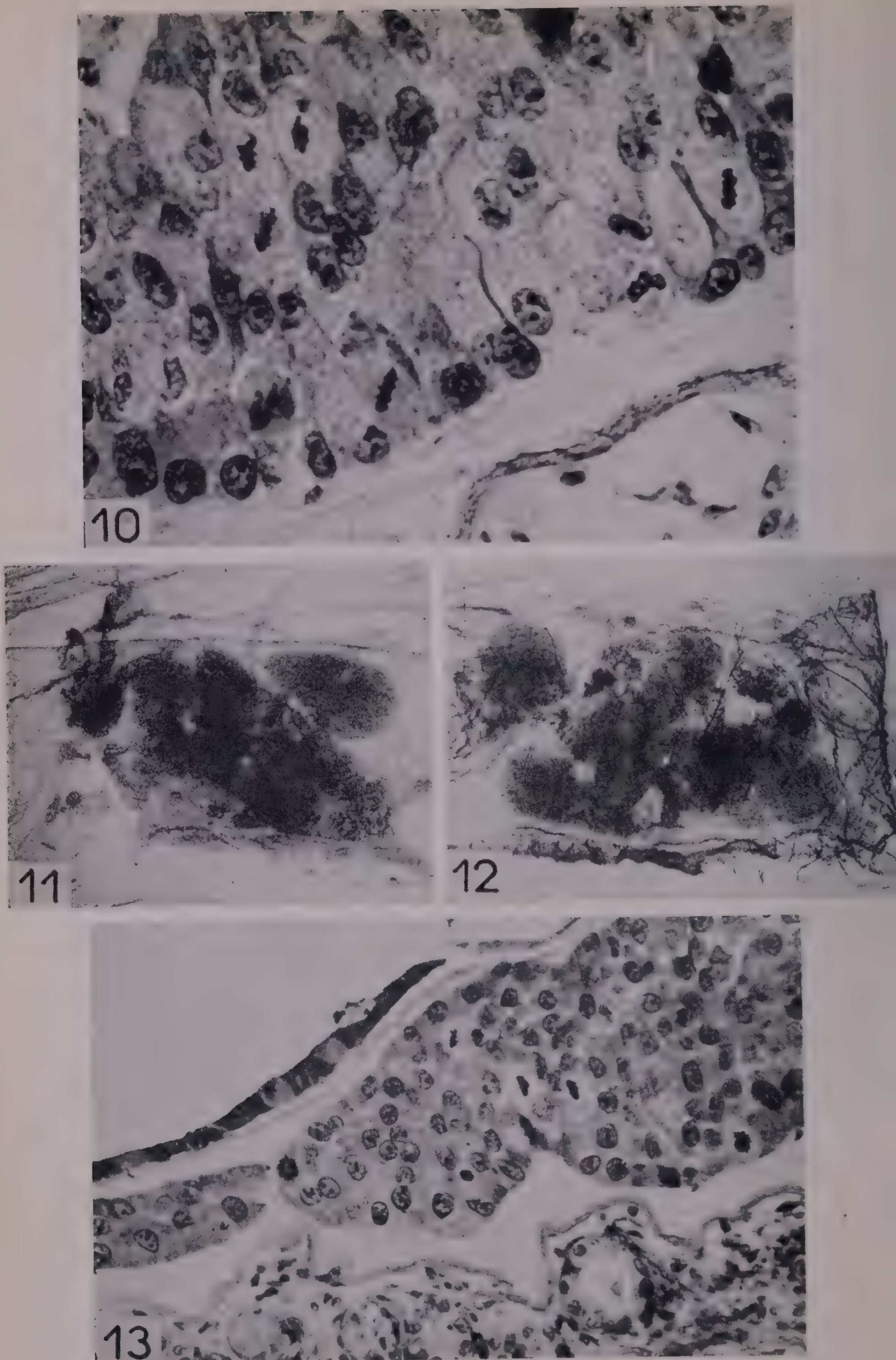

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Planche 4

the absence of the organ itself, is enough to ensure the survival and growth of the tumour.

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EXPLICATION DES PLANCHES

PLANCHE 1

FIG. 1. Migration des cellules du sarcome S. 180 le long de la membrane vitelline qui limite, en haut et en bas, l'explant de mesonephros. Pénétration des cellules entre les tissus du mesonephros. × 200.

FIG. 2. Formation d'un nodule de cellules KB sous la membrane vitelline, aux dépens des tissus du mesonephros. La membrane vitelline, qui adhère intimement aux explants pendant la culture, se décolle souvent au cours des opérations de la technique histologique. ×205.

FIG. 3. Culture d'un chondrosarcome au contact du mesonephros. Nombreux groupes de chondroblastes en voie de différenciation. A gauche, une rangée de petites cellules, au contact du mesonephros, propage la tumeur à la périphérie du nodule. ×110.

FIG. 4. Invasion de toute l'épaisseur du mesonephros par les cellules de la souche HeLa. Les tubes urinaires, encore bordés par leur épithélium très aminci, sont complètement entourés par les cellules de la tumeur. ×210.

PLANCHE 2

Cultures de cellules cancéreuses, séparées du mesonephros par une membrane.

FIG. 5. Vue générale d'un nodule de cellules HeLa, séparées du mesonephros par la membrane vitelline. Vers la gauche, essaimage de la tumeur et formation d'un petit nodule. ×120.

FIG. 6. Nodule de cellules KB, dans le compartiment supérieur de la membrane vitelline. Le mesonephros se trouve dans le compartiment inférieur. Le large espace entre la membrane et le mesonephros est un artefact dû à la technique histologique. En culture, le mesonephros adhère intimement à la membrane. ×160.

FIG. 7. Un aspect fréquent des rapports entre la culture et le mesonephros. Les cellules cancéreuses adhèrent intimement à la surface de la membrane, qu'elles poussent en se multipliant vers l'intérieur des tissus du mesonephros. Ainsi se constitue une sorte de suçoir. ×175.

PLANCHE 3

FIG. 8. Colonies de cellules KB dans une pochette superposée au mesonephros. Noter l'adhérence des cellules aux deux membranes qui les limitent et leur disposition régulière. ×340.

FIG. 9. Colonie de cellules Fogh, montrant une activité mitotique intense et une disposition épithéliale en palissade. ×320.

PLANCHE 4

FIG. 10. Détail d'une colonie de cellules KB s'appuyant sur la membrane vitelline (face fibreuse). Noter la disposition épithéliale et la grande activité mitotique de ces cellules. × 680.

FIGS. 11 et 12. Vue générale, par transparence, de deux cultures vivantes de la souche KB dans une poche de la membrane vitelline superposée au compartiment du mesonephros. Toutes les masses

sombres, plus ou moins rattachées les unes aux autres, aux contours arrondis, correspondent aux cultures de cellules cancéreuses. ×15.

Fig. 13. Le bord d'une culture de cellules HeLa, montrant la croissance de la colonie par la multiplication active de ses cellules et par détachement de colonies-filles. (Au-dessus de la membrane vitelline supérieure, on voit un cordon très sombre de cellules cancéreuses qui ont fait irruption par une déchirure de la membrane.) ×380.

Planche 5

Accroissement périphérique des cultures.

Fig. 14. Bord d'une colonie de KB, montrant la grande activité mitotique de ses cellules. Souche KB. ×440.

Fig. 15. Constitution d'un nodule secondaire à partir de cellules détachées de l'amas principal. Souche KB. ×560.

Planche 6

Croissance et migration des cellules du sarcome S 180, avec membrane interposée.

Fig. 16. Vue d'ensemble, montrant l'un des explants initiaux et les nappes de cellules migrant sur les bords entre les deux feuillets de la membrane vitelline. ×110.

Fig. 17. Région médiolatérale du même explant, à un plus fort grossissement. Les cellules du sarcome S 180 ont tendance à déserter la région centrale; elles se pressent contre la membrane vitelline inférieure et vers les bords latéraux de l'explant. ×300.

Fig. 18. La membrane de séparation entre l'explant du sarcome S 180 et le mesonephros a été traitée par la trypsine. Les cellules tumorales traversent la membrane partiellement digérée et font irruption entre les tissus du mesonephros. ×225.

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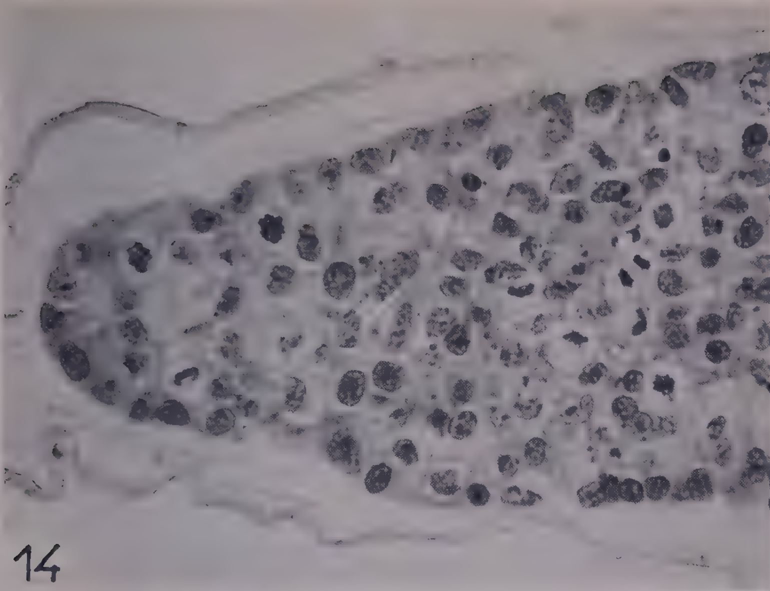

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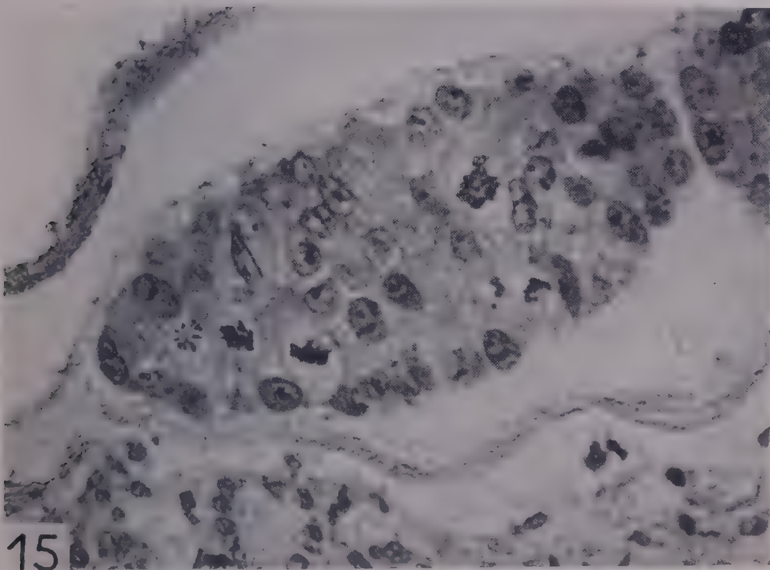
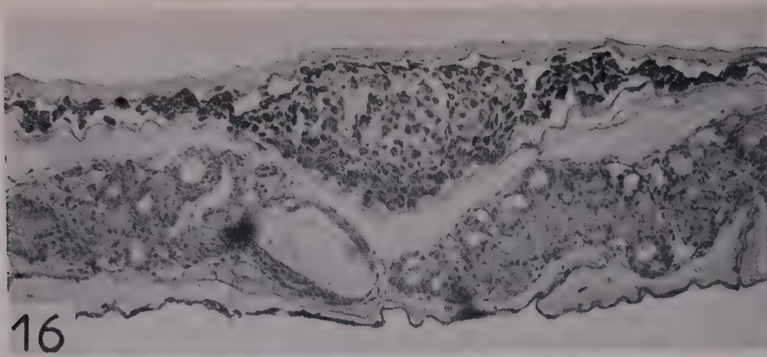
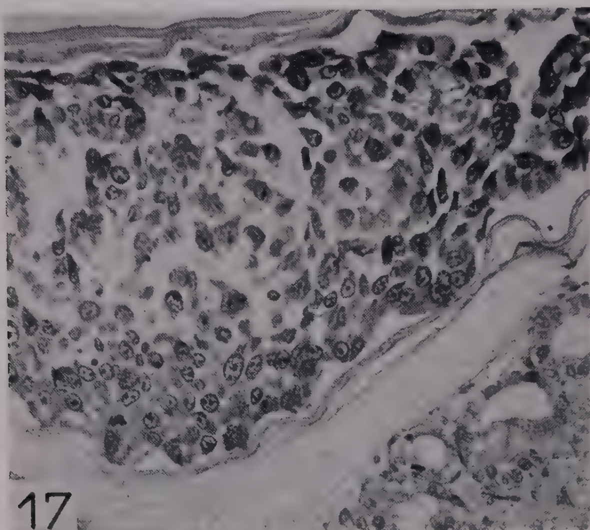


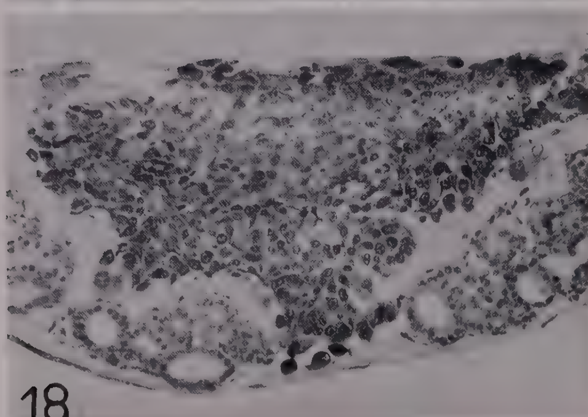
15

ETIENNE WOLFF *et* EMILIENNE WOLFF

Planche 5







ETIENNE WOLFF *et* EMILIENNE WOLFF

Planche 6

Index of Authors

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